VOLUME 9 March 1960 NUMBER 2

THE AMERICAN JOURNAL OF

Tropical Medicine and Hygiene

Combining The American Journal of Tropical Medicine and The Journal of the National Malaria Society

OFFICIAL ORGAN OF
THE AMERICAN SOCIETY OF TROPICAL MEDICINE AND HYGIENE

PUBLISHED BIMONTHLY BY
THE WILLIAMS & WILKINS COMPANY
BALTIMORE 2, MARYLAND, U.S.A.

© Copyright 1960, By The American Society of Tropical Medicine and Hygiene
Made in U.S.A.

THE AMERICAN JOURNAL OF

Tropical Medicine and Hygiene

Editor-in-Chief
PAUL C. BEAVER
1430 Tulane Avenue
New Orleans 12, Louisiana

Associate Editor
MARTIN FROBISHER
374 Ruckman Road
Closter, New Jersey

Editor, Tropical Medicine and Hygiene News
WILLIAM B. DEWITT, National Institutes of Health
Bethesda 14, Maryland

Editorial Board

WILLIAM McD. HAMMON
JOHN A. LOGAN
HARRY MOST
LLOYD E. ROZEBOOM
ALBERT B. SABIN
LEON H. SCHMIDT
THOMAS H. WELLER
MARTIN D. YOUNG

INFORMATION FOR SUBSCRIBERS

THE AMERICAN JOURNAL OF TROPICAL MEDICINE AND HYGIENE is issued bimonthly, appearing in January, March, May, July, September, and November. One volume is issued per year.

Correspondence concerning business matters, subscriptions and orders for reprints, should be addressed to The Williams & Wilkins Company, 428 East Preston Street, Baltimore 2, Maryland, U. S. A.

Manuscripts should be sent to Dr. Paul C. Beaver, 1430 Tulane Avenue, New Orleans 12, Louisiana. *Books for Review* should be sent to Dr. Martin Frobisher, 374 Ruckman Road, Closter, New Jersey.

New subscriptions and renewals are entered to begin with the first issue of the current volume. Should any issue of the current volume be out of print at the time the subscription order is received, the pro rata value of such numbers will be refunded to the subscriber.

Subscriptions should be renewed promptly—To avoid a break in your series, subscriptions should be renewed promptly. The publishers cannot guarantee to supply back issues on belated renewals.

Change of address: Publisher must be notified 60 days in advance. Journals undeliverable because of incorrect address will be destroyed. Duplicates can be obtained (if available) from the publisher at the regular price of single issues.

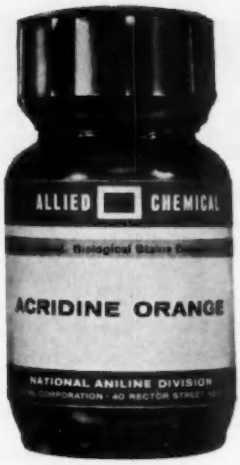
OFFICERS OF THE

American Society of Tropical Medicine and Hygiene

President	*Councilors:*	*Term Expires*
WILLARD H. WRIGHT	JOHN M. HENDERSON	1960
President-Elect	J. ALLEN SCOTT	1960
WILLIAM W. FRYE	PAUL F. RUSSELL	1961
Vice-President	JOSEPH E. SMADEL	1961
G. ROBERT COATNEY	MARSHALL HERTIG	1962
Secretary-Treasurer	JOHN E. LARSH, JR.	1962
ROLLA B. HILL	ARNOLDO GABALDON	1963
3575 St. Gaudens Road	HARRY MOST	1963
Miami 33, Florida		

Individuals interested in any phase of tropical medicine and hygiene, including all arthropod-borne diseases, medical care and human welfare are eligible for membership. Dues are $10.00 a year and include subscription to the American Journal of Tropical Medicine and Hygiene. An application blank is bound into this issue of the Journal for the use of interested individuals. Before being submitted to the Secretary, each application must be signed by two active members of the Society and a check for $10.00 (U.S. banks preferred) must accompany the application.

Published bimonthly by The Williams & Wilkins Co., 428 East Preston Street, Baltimore 2, Md., U.S.A.
Subscription price $10.00, single issue $2.00
Second-class privileges authorized at Baltimore, Md.



National ACRIDINE ORANGE

for use in

FLUORESCENCE MICROSCOPY

REGISTER FOR NEW DATA ON NATIONAL FLUORESCENT STAINS

Recognizing the growing importance of fluorescence microscopy, National Laboratories are engaged in a fundamental research program for the development of additional fluorescent stains. We shall be pleased to send you information on the results of this work, as it becomes available, if you will indicate your interest by sending your name and address to the Technical Director, Biological Stain Department, NATIONAL ANILINE DIVISION, 40 Rector St., New York 6.

This National Biological Stain is finding increasing favor in fluorescent staining techniques developed for exfoliative cytology, gynecological diagnoses and in the diagnosis of cervical and vaginal carcinoma and other conditions where fast, precise diagnoses are greatly to be desired.

Stocks of National ACRIDINE ORANGE (Code 408) are now carried by leading medical supply houses who will be pleased to supply your needs promptly.

NATIONAL ANILINE DIVISION
40 RECTOR STREET, NEW YORK 6, N. Y.
Atlanta Boston Charlotte Chicago Greensboro Los Angeles
Philadelphia Portland, Ore. Providence San Francisco
In Canada: ALLIED CHEMICAL CANADA, LTD., 100 North Queen St., Toronto 18
Distributors throughout the world. For information:
ALLIED CHEMICAL INTERNATIONAL • 40 Rector St., New York 6, N. Y.

Allied Chemical

SMITH SPRAYERS

"For 70 Years Choice for Quality the World Over"

FOR MOSQUITO & MALARIA CONTROL

For Residual House Spraying or Larviciding Operations. Herculoy Silicon Bronze Sprayers are available in 4 gallon and smaller sizes and Armco Zinc-grip steel sprayers are also available in smaller sizes.

E-Z Knapsack Sprayer

HEAVY SEAMLESS BRASS PUMP QUICKLY REMOVED.
LARGE 4" OPENING FOR QUICK FILLING REMOVABLE BRASS STRAINER
TRIGGER SHUT-OFF LOCKS OPEN.
BRASS ADJUSTABLE NOZZLE FOR FOG MIST, COARSE OR LONG DISTANCE SPRAY.
E-Z
KNAPSACK SPRAYER
TUBULAR STEEL.
PISTON — HEAVY OIL-PROOF RUBBER FOR LONG SERVICE. EASILY REPLACED.
DASH AGITATOR
VENTILATED AND FORM FITTING BACK.
TANK MADE OF ARMCO ZINC GRIP STEEL OR MANGANESE BRASS. ARMCO IS FAR SUPERIOR TO ANY GALVANIZED METAL.
1-PIECE BRASS PUMP CUP.
AIR-COOLED TANK
5-GALLON TANK

One in case.
Shipping weight—20 lbs.

Maintains continuous high pressure, with only easy slow pumping. Pump entirely of brass to stand long hard usage. Tank solid copper or Armco zinc-grip steel. Equipped with best grade, high-pressure hose, 2-foot brass extension pipe with adjustable brass or fixed type nozzle. New shut-off control may be locked open for continuous spray. Capacity 4 to 5 gallons. Has form-fitting ventilated tank, keeping the back dry and comfortable. Carrying handle; and dasher type agitator which keeps liquid thoroughly mixed. Brass liquid strainer.

MANY OTHER STYLES— SEND FOR CATALOG

on our complete line of hand, continuous, compressed air, knapsack, bucket, wheelbarrow and barrel sprayers. Also hand and crank powder insecticide dusters. We are the originators of sprayers and our products are of outstanding quality in engineering, modern design and performance.

D. B. SMITH & COMPANY, 432 MAIN ST., UTICA 2, NEW YORK, U. S. A.
ESTABLISHED 1888

THE AMERICAN JOURNAL OF
TROPICAL MEDICINE AND HYGIENE

Vol. 9 *March 1960* *No. 2*

CONTENTS

Once upon a time. Presidential address. L. W. HACKETT 105

Studies on trachoma. I. Isolation and identification of strains of elementary bodies from Saudi Arabia and Egypt. E. S. MURRAY, S. D. BELL, JR., A. T. HANNA, R. L. NICHOLS, AND J. C. SNYDER 116

The nature of the gastrointestinal lesion in Asiatic cholera and its relation to pathogenesis: a biopsy study. E. J. GANGAROSA, W. R. BEISEL, CHANYO BENYAJATI, H. SPRINZ, AND PRAPONT PIYARATN 125

Survival, and protection against chlorination, of human enteric pathogens in free-living nematodes isolated from water supplies. SHIH LU CHANG, GERALD BERG, NORMAN A. CLARKE, AND PAUL W. KABLER 136

Phycomycosis (mucormycosis) in Indonesia—description of a case affecting the subcutaneous tissue. LIE KIAN JOE, NJO-INJO TJOEI ENG, SUTOMO TJOKRONEGORO, AND CHESTER W. EMMONS 143

A combination of amodiaquin and primaquine (Camoprim) in the prevention and cure of sporozoite-induced Chesson strain *vivax* malaria. KENNETH O. COURTNEY, ROBERT HODGKINSON, REX RAMSEY, AND MEIGS HAGGERTY 149

Humatin in intestinal amebiasis. S. BELL AND A. W. WOODRUFF 155

Sensitivity of Lebanese and Syrians to toxoplasmin and to a similar antigen prepared from *Leishmania tropica*. KAMAL ABOU DAOUD AND CALVIN W. SCHWABE 158

Some quantitative data on the growth of *Entamoeba histolytica* from single-cell isolations in microcultures. CHARLES W. REES, IRIS D. KEY, AND JAMES G. SHAFFER 162

Intestinal protozoa and helminths in school children of Dacca, East Pakistan (East Bengal). ROBERT E. KUNTZ 168

Intestinal parasites in the Panama Canal Zone. G. E. COSGROVE 173

The ingestion of bacteria and of altered bacterial structures by *Entamoeba histolytica* in Shaffer-Frye and in modified Shaffer-Frye media. JAMES G. SHAFFER AND IRIS KEY 175

Reduction in the prevalence of clinical filariasis in Tahiti following adoption of a control program. H. N. MARCH, JACQUES LAIGRET, JOHN F. KESSEL, AND BEN BAMBRIDGE 180

Use of a hemagglutination test in visceral larva migrans. RODNEY C. JUNG AND GUILLERMO PACHECO 185

Human sparganosis in Mississippi: report of two cases. T. J. BROOKS, JR., W. F. HUTCHISON, T. J. SAFLEY, AND T. G. ROSS 192

On the virulence of the geographic strains of *Schistosoma japonicum*. S. Y. LI HSÜ AND H. F. HSÜ 195

The effects of low concentrations of sodium pentachlorophenate on the fecundity and egg viability of *Australorbis glabratus*. LOUIS OLIVIER AND WILLARD T. HASKINS 199

Transactions of the American Society of Tropical Medicine and Hygiene. Eighth Annual Meeting, Indianapolis, Indiana, October 29–31, 1959 206

Books Received 222

Book reviews: *The Ecology of the Medical Student*, by Lee and Glaser; 1959—DDT. *The Insecticide DDT and its Significance*, by Muller, ed'r. *Vol. II. Human and Veterinary Medicine*, by Simmons, ed'r.; *The Human Integument*, by Rothman, ed'r.; *Annual Review of Entomology*, by Steinhaus, ed'r. and Smith, assoc. ed'r. 222

Correction 227

Eliminate

PINWORMS
ROUNDWORMS

Without staining • vomiting • enemas
nausea • fasting • laxatives

Available as . . .

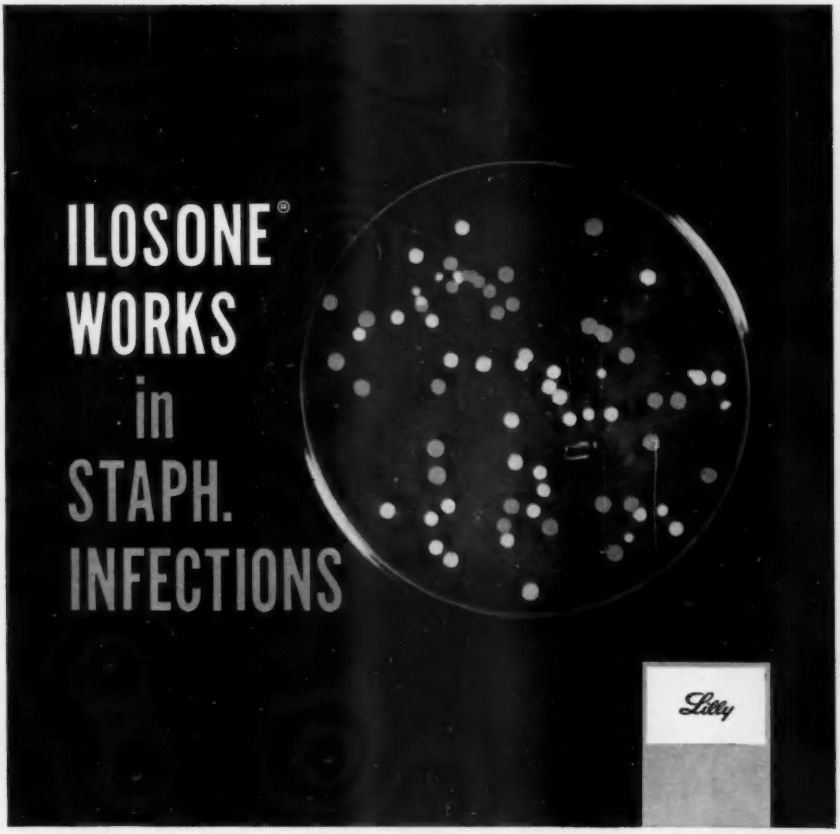
'ANTEPAR' SYRUP Piperazine Citrate, 100 mg. per cc.

'ANTEPAR' TABLETS Piperazine Citrate, 500 mg., scored

'ANTEPAR' WAFERS Piperazine Phosphate, 500 mg.

Literature and patient instruction sheets available on request

BURROUGHS WELLCOME & CO. (U.S.A.) INC., Tuckahoe, New York



"In our hands it has been particularly helpful in the treatment of staphylococcic disease."[1]

In difficult staph. infections, a decisive response may be obtained with Ilosone in a high percentage of cases.

In a study[1] of 105 patients, sixty-four of whom had *Staphylococcus aureus* infections, good results were obtained with Ilosone in 94 percent. Ten subjects had previously failed to respond to other forms of chemotherapy. The authors concluded that Ilosone ". . . is useful in treatment of a number of common infections and has been effective in treatment of a number of less common and more serious infections. . . . In our hands it has been particularly helpful in the treatment of staphylococcic disease."

Ilosone is available in **Pulvules®**, 125 mg. and 250 mg.; Lauryl Sulfate **125 Suspension,** 125 mg. (base equiv.) per 5-cc. tsp.; and Lauryl Sulfate **Drops,** 5 mg. (base equiv.) per drop. Usual dosage for adults and children over fifty pounds is 250 mg. every six hours.

1. Smith, I. M., and Soderstrom, W. H.: J. A. M. A., *170*:184 (May 9), 1959.

Ilosone® (propionyl erythromycin ester, Lilly)

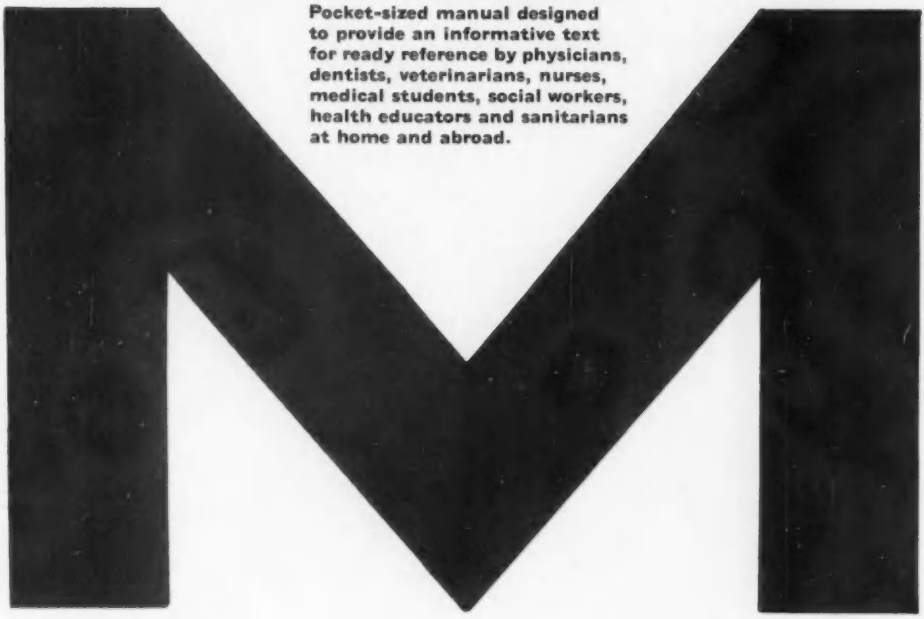
ELI LILLY AND COMPANY • INDIANAPOLIS 6, INDIANA, U.S.A.

032535

ANNOUNCING . . . THE NEW 9TH EDITION OF CONTROL OF COMMUNICABLE DISEASES IN MAN

An Official Report of the American Public Health Association

Pocket-sized manual designed to provide an informative text for ready reference by physicians, dentists, veterinarians, nurses, medical students, social workers, health educators and sanitarians at home and abroad.



- Every chapter has had material revision, to take advantage of new information developed in the past five years. The particular diseases which have undergone greatest revision include anthrax; histoplasmosis; influenza; poliomyelitis; rabies; rubella; tetanus; toxoplasmosis.
- New chapters have been added on aseptic meningitis syndrome (its relation to non-paralytic poliomyelitis); acute upper respiratory disease (incorporating the former chapter on the common cold); loaiasis; staphylococcal infections (emphasizing the occurrence of such infections in hospital practice); four new chapters on arthropod-borne infections (a general summary of this group of virus infections, the encephalitides, arthropod-borne fevers and arthropod-borne hemorrhagic fevers).
- Index has been enlarged for easy reference.

Edited by international authorities:

John E. Gordon, M.D., *Chairman*

Gaylord W. Anderson, M.D.
Joseph A. Bell, M.D.
Harold W. Brown, M.D.
E. Gurney Clark, M.D.
John H. Dingle, M.D.
Chester W. Emmons, Ph.D.
William W. Frye, M.D.
William McD. Hammon, M.D.
Arthur C. Hollister, Jr., M.D.
Robert F. Korns, M.D.
Alexander D. Langmuir, M.D.
Philip E. Sartwell, M.D.
Joseph E. Smadel, M.D.
Franklin H. Top, M.D.

235 pp. cloth cover $2.50: paper cover $1.00
Book Service, American Public Health Association
1790 Broadway, New York 19, N. Y.

a rapid return to health with

CAMOQUIN®

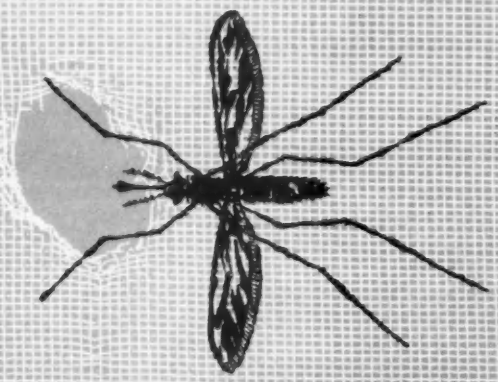
SINGLE-DOSE ANTIMALARIAL

A single three-tablet dose of CAMOQUIN hydrochloride (amodiaquin hydrochloride, Parke-Davis) reduces fever, clears the blood stream, and relieves other symptoms —often within 24 hours. Ideal for mass treatment because of its economy and fast action. CAMOQUIN is also valuable as a suppressive agent.

Supplied: In packages of 3 tablets of 0.2 Gm. each, and in bottles of 1000 tablets.

50166

PARKE, DAVIS & COMPANY
DETROIT 32, MICHIGAN



A sociological and medical study of bejel. . .

Hudson: NON-VENEREAL SYPHILIS

A study of the disease bejel in sociological and medical terms, by a world authority in treponemal infections. Advocates the recognition of syphilis and yaws as components of world-wide treponematosis, with bejel as an example of endemic syphilis which stands between them and draws them together. Of interest not only to the specialist in syphilis and tropical medicine, but also to the general physician, the public health officer, the lay health worker, the biologist, the sociologist, the anthropologist, and the student of geography of diseases.

Contents include: Treponematosis, the "Twisted Thread" Infection; Historical Perspective of Treponematosis; Bejel and Franji; Three Social Groups; Sex and Syphilis in the Three Social Groups; Incidence of Treponematosis; Venereal Lesions and Infections; Early Stage; Clinical Picture of Early Stage; Transmission of Bejel; Latent Period; Late Stage; Clinical Picture of Late Stage; Special Features of Late Stage; Effect of Bejel on Next Generation; Serology of Bejel (By Maunu Tuomioja, M.D., Helsinki); Microscopic Pathology of Bejel (By Richard E. Olsen, M.D., Pontiac, Michigan); Experimental Pathology of Bejel (Excerpts from T. B. Turner, M.D. and D. H. Hollander, M.D., Baltimore); Treatment of Bejel; Significance of Bejel; A New Social Hygiene.

By ELLIS HERNDON HUDSON, *M.D., D.T.M. & H. (Lond.), F.A.C.P., Emeritus Professor and Director of Health, Ohio University*

212 pp., 91 figs. (1957) • Price: **$7.00**

Publishers of Medical and Scientific Books and Periodicals

SANS TACHE

WILLIAMS & WILKINS COMPANY

BALTIMORE 2, MD.

SHOP BY MAIL

THE WILLIAMS AND WILKINS COMPANY
Baltimore 2, Md.

Please send the following on approval:

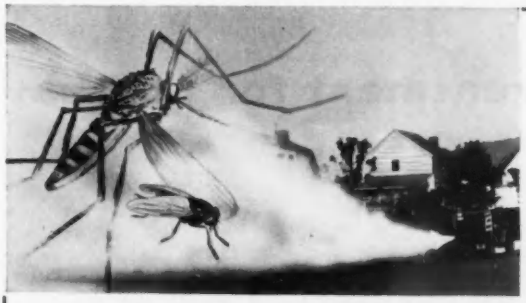
Name _____ (Please print)

Address _____

City _____ Zone _____ State _____

☐ Payment enclosed. ☐ Bill me.

Shopping by mail is an easy, time-saving way to select books for your personal library.



MOSQUITOES, FLIES AND
OTHER INSECT PESTS

DESTROYED ON CONTACT

TIFA

NOW SUITABLE FOR OPERATION ON PROPANE OR GASOLINE

Best by test for over 15 years . . . on the dump, over pools, in suburbs, play areas, stockyards, everywhere that insects swarm. TIFA, lowest of all in operating and maintenance costs, rapidly covers large areas with true clean fog. Maximum lethal efficiency with newest or older type insecticides. One-man operation. Starter-generator optional.

TODD INSECTICIDAL FOG APPLICATOR
OVER 15 YEARS successful world-wide performance in Public Health programs of more than 5,000 communities.
Carrying Underwriters' Listing Seal No. MH 4162.

Write for illustrated folder and official comments.

TODD SHIPYARDS CORPORATION
PRODUCTS DIVISION
SALES AND SERVICE DEPARTMENTS Columbia and Halleck Streets, Brooklyn 31, N. Y.
PLANT, SALES AND SERVICE Houston, Texas
Sales and Service Agencies throughout the Western Hemisphere

THE AMERICAN SOCIETY OF TROPICAL MEDICINE AND HYGIENE

APPLICATION FOR MEMBERSHIP

To the Membership Committee:

The following individual interested in tropical medicine and hygiene is proposed for active membership

(TYPE OR PRINT)

Name ..... (*Last*) ..... (*First*) ..... (*Middle*)

Mailing Address: Street ..... City .....

State ..... Country .....

Date of Birth ..... Place .....

Present Position .....

Special Field of Interest .....

Education: Institution (Graduate and Postgraduate) Degree Date

.....

.....

.....

Attach to the application a list of professional positions held, membership in scientific societies, and a list of major publications.

* Proposed by:

(1) ....

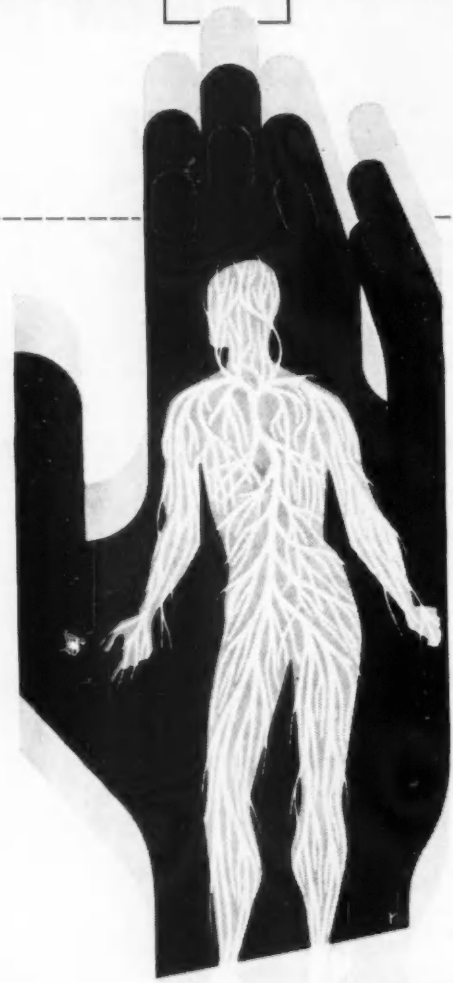
(2) ..... Signature of Applicant

* Two active members must sign this application. Date .....

Return this application to Dr. Rolla B. Hill, Secretary-Treasurer, 3575 St. Gaudens Road, Miami 33, Florida

(*Check for $10.00 (U. S. banks requested) must accompany this application.*)

The new ICI treatment for Leprosy



Advantages of 'Etisul'

1 Chemotherapeutic action —
Rapid and pronounced.

2 Safety —
Side effects minimal, regardless of age or sex of the patient.

3 Psychologically sound
in its method of administration.

4 Mass treatment —
DDS and 'Etisul' may be combined with advantage in mass treatment.

'Etisul' (percutaneous) is presented as a cream for inunction in unit packs containing 5 grammes of active agent.

Literature and further information available on request.

References: Lep. Rev. (1959), 1, 61-72
Ibid. (1959), 3, 141

Etisul
Trade Mark

(PERCUTANEOUS)

Diethyl-Dithiolisophthalate (I.C.I. Compound 15688)

IMPERIAL CHEMICAL INDUSTRIES LIMITED
Ph.942.0 PHARMACEUTICALS DIVISION WILMSLOW CHESHIRE ENGLAND

ICI

ONCE UPON A TIME

Presidential Address

L. W. HACKETT

Justice Frankfurter has said there are two myths which have always captivated the human race, that of a Utopia in the unforeseeable future, and that of a Golden Age in some remote epoch of the past. In the dynamic field of world health we foster the same myths but shorten the time span. To a Society like ours made up in its majority of young, imaginative and forward-looking persons, the Utopia is just around the corner—a happy if somewhat overcrowded world freed forever from malaria, tuberculosis, smallpox, yaws, leprosy and syphilis. But I, who have reached the age of retrospection, find pleasure in looking back to a Golden Age no longer ago than my own youth, when public health, and especially international public health, were at the starting line, and progress was about to make a great leap forward, as the Chinese say, into a new, rich and virgin field. So I think it more appropriate tonight to review the record than to attempt to scan the future—more appropriate and more circumspect.

My first chief, Wickliffe Rose, who in 1913 organized the International Health Commission of the Rockefeller Foundation, was impressive in action and endowed with extraordinary vision and courage, but he was modest and self-effacing in his public relations, and disliked predictions. The sanguine neophytes on his staff, with all the confidence of youth and inexperience, never doubted their ability to conquer yellow fever, suppress malaria, and purge the entire "wormy world." But Rose was prudent. "We are only safe," he would admonish them, "in talking about the past." Rose was forgetting that these young doctors fresh from medical school had no past; they had nothing to talk or think about but the future. I was one of those youths, among the first to be appointed to the staff of the International Health Commission, and since that fateful conversation with Mr. Rose in April, 1914, I have accumulated enough past to talk about. My purpose tonight is to delve among the roots of the great, spreading international health movement to uncover its modest beginnings in Rose's International Health Commission, the IHC, known in successive epochs as the International Health Board (IHB) and the International Health Division (IHD).

Rose had a program for action so simple and so reasonable that it is astonishing it never had been attempted before. The world had always been defenceless against recurring invasions of certain great and destructive diseases whose forays and infiltrations from their endemic seedbeds were a constant threat to every country on earth, in spite of all the barricades and quarantines which were erected in times of trouble, but which were always as ineffectual as they were harsh and oppressive. Rose's design was to wage a coordinated mass attack on some of these dangerous but preventable diseases on their home grounds, wherever they were found to be endemic and uncontrolled. Rose proposed to offer any government in this situation the technical and financial collaboration of the IHC, a proposition without parallel in history, and one which had its remarkable aspects, considering the disparities in age, size and wealth between the two parties. Dr. William H. Welch, in accepting a position on the Commission, said, "both the purpose of the gift and the opportunity it creates are unique in the annals of preventive medicine."

Actually, the IHC was not without a prototype in its philanthropic plan to extend a helping hand around the world to fight the aggressive diseases. Charles IV of Spain despatched a commission to disseminate the knowledge and application of Jenner's discovery of cowpox inoculation for the prevention of smallpox. This procedure had reached France, Spain and the U. S. by 1800, and Charles IV undertook to send out a scientific mission to go completely around the world carrying the protective virus "to all Spanish lands and all other races and peoples en route." The royal order was given August 3, 1803, and a military surgeon, Dr. Francesco Javier de Balmis set sail from La Coruña for America on November 30, accompanied by 3 doctors, 2 assistants, 3 male nurses and the Directress of the local orphan asylum in charge

of 22 nursing babies to conserve the virus during the long journey, passaging it from arm to arm. All arrived safely in Venezuela, where the expedition divided in two, one part going south to cover South America while de Balmis himself, after attending to the needs of the Caribbean area and Central America, crossed Mexico and sailed from Acapulco to the Philippines, where he arrived with the virus still alive and potent. Continuing on around the world he reached Lisbon August 15, 1806, two years and nine months after leaving La Coruña. The other half of the expedition was not so fortunate. It was shipwrecked at the mouth of the Magdalena River, but all the babies were saved. The group visited Colombia, Ecuador and Peru, and was preparing to go on to Chile and Buenos Aires when they learned in Lima that the vaccine had already reached the Rio de la Plata.

This story was given to me by Dr. Rafael Schiaffino, the medical historian of Uruguay, and I am sorry I cannot give you the further details we should all like to know about this picturesque expedition—how the 3 male nurses were able to provide nourishment for 22 nursing babies, what happened to the little ship and its crew as this gang of young delinquents reached the mobile and destructive age of three, and how the virus was kept alive for almost 3 years with only 22 passages. In any case, the fact is that a scientific commission had encircled the globe to promote a general attack on one of the great pestilential diseases of the age and to instruct backward and isolated peoples in the use of a new preventive measure of the first importance.

The great epidemics continued to ravage Europe and America, and often swept around the world, but I cannot find that any coordinated action was ever again taken to curb them until Rose appeared on the international scene. The great powers, including the U. S., did not, it is true, suffer these blows in silence; after each major visitation of plague, cholera, yellow fever, smallpox or typhus, they held an international sanitary conference, usually in Paris, and kept up this practice for over 50 years without visible effect on the incursions of the epidemics. It was like the first installation of foghorns on the California seaboard, which the coastal inhabitants are reputed to have called a futile gesture and a waste of money, since the fog kept rolling in just as before. The governments of the 19th century could not agree on any effective plan for a joint campaign against these common enemies because any suggestion that an international agency, even of their own creation, should be permitted to make inspections and carry out measures behind their frontiers was considered an intolerable derogation of national sovereignty and an affront to national pride. But now a private organization, whom no one had ever heard of, with no foreign experience and as yet without a staff, assumed that these same governments would invite it to enter their countries and help them combat certain diseases which they had failed to curb, and thus establish a united front against them all over the world. Rose was resolved to cooperate only with governments, and this was breaking ground in a number of fields; it was new to philanthropy, to international diplomacy and to public health. No one has ever accused Rose of being either naive or foolhardy, but the prospect in 1913 was one which might have daunted any valiant soul. For not only, as it turned out, was there all too scanty knowledge of the epidemiology of the diseases to be attacked, but as these gradually became better understood, the drugs and tools and other measures available proved very ineffective to control, not to speak of eradicating any of them.

But perhaps Rose's most serious handicap was the lack of any trained and experienced personnel, and this was so because there was no career in public health; practicing physicians doubled as health officers in their spare time. Rose had foreseen this major obstruction to his program long in advance. When the IHC was no more than a scheme presented to the Trustees of the Rockefeller Foundation (RF) at their first meeting, he asked the General Education Board to survey existing facilities in the U. S. for the postgraduate education of doctors in the principles and techniques of hygiene, a training which he needed not only for his own staff, but for all the countries where its work would lead to constructive developments in public health. It turned out that there were no such facilities; evidently a new kind of professional school was needed which did not exist. The RF offered at once to assist universities to develop such institutions for training and research in public health. The first of these, planned and organized by Dr. Welch was the Johns Hopkins School

of Hygiene and Public Health which enrolled its first class of 17 students in the fall of 1918. This was followed by grants to Harvard, Toronto, London and in time to a large number of schools all over the world. A majority of the students in the early years were foreign fellows sent by the IHC and its successors, and this was one of the most acceptable and constructive forms of international collaboration the RF ever undertook. The fellowship program acquired volume as the projects multiplied, until in the end the International Health Division had spent some 7 million dollars on what Rose called an investment in leadership. These great schools and the thousands of health workers whom they trained, constituted an educational force which had a profound influence on the well-being of mankind. The IHC thus set on foot a movement which resulted in a new profession of full-time health officers, in the multiplication of health services to employ them, and in the creation of schools to train them. Rose had set his sights as high as this from the beginning and always obtained the support he needed from the RF, running into many millions of dollars. He wrote at the end, "Public enlightenment, government machinery, and technical education and research are bound up in a sure sequence."

In 1914, when the work got started, this evolution had not even begun. Finding personnel was no small task. The U. S. was soon in the war and he had to scrape the barrel for candidates. These could be given at that time no proper field training in the U. S. to prepare them for a foreign program; they were equipped with only the conventional medical education of 50 years ago, before Abraham Flexner's report of 1910 had had time to improve it. Rose did not, for all this, slow down the expansion of his foreign program; within 5 years he had made agreements for projects with 42 governments, and 3 years later, when he resigned to take up other work, the number had increased to 63. Recruitment of staff never caught up in Rose's time with the invitations which came flooding in from all over the world. Since he could not expect training or experience, Rose chose his men for character and bent. They were sent overseas as soon as possible and with a minimum of briefing to some previously established station, and then on to their own destination in a land whose language, politics and ways were no less strange to them than its diseases. When I look back upon this picture, I marvel at Rose's serene confidence both in his ballooning program which seemed always about to get out of hand but never did, and in his young doctors who knew very little of the world and its peoples, or of tropical medicine and hygiene, but who rarely let him down.

The field staff learned by doing; education is the accumulation of experience, not of facts, and as Oscar Wilde said, experience is the name we give our mistakes. I feel now that our ignorance and inexperience were paradoxically an advantage in the beginning when there was not a great deal of existing knowledge to be drawn upon from any source. My early experience was in Latin America, and when the local doctors with whom we worked found out that we did not look down upon them as ignoramuses but eagerly listened to what they had to tell us, their suspicions and defense armor soon melted away and a happy relationship of genuine and fruitful reciprocity was established. They taught us their language, politics and folkways, and what they knew about tropical disease; they got from us our kind of practical approach to problems, our lack of frustration in the face of hypothetical difficulties, and the funds to carry out demonstrations and experiments on a significantly large scale. We were all empiricists, learning what we could from observation and experience, and Rose and the rest of us refused to be dismayed by presumptive obstacles.

For one thing, Rose started with hookworm disease, a sound choice as it turned out—the best he could have made. The hookworm work never led to such dramatic victories as malaria nor to such fruitful discoveries as yellow fever, but as an entering wedge for a long-term public-health program, it was far superior to either. There were no mysteries about the nature of the hookworm or the way it spread; it was large enough for the victims to see without a microscope, it infested an enormously wide belt, running clear around the earth, which contained more than half the population of the world, and it was vulnerable to treatment and sanitation. The Old World tropics were all colonial possessions in 1914 except for Liberia, Ethiopia and Siam, and the British no sooner learned of Rose's program than they invited him to begin in any or all of their tropical colonies. Thus the question as to how the great Powers would react to Rose's

initiative was answered at once to his satisfaction. Furthermore, he was not going into this thing blindly; he had just carried out from 1910 to 1914 a sort of pilot experiment in hookworm control on a large scale which had revealed both the obstacles he could expect to meet and the results he might hope to achieve. Wickliffe Rose, a layman and distinguished Southern educator, had been wisely chosen rather than a medical man to organize the hookworm campaign of the Rockefeller Sanitary Commission in the Southern States. The medical and sanitary problems were so simple that they could be handled by young doctors and sanitary engineers with a minimum of special training, but the promotional and educational aspects of hookworm control were subtle and difficult, calling for a leader experienced in methods of teaching and in the ways of the South.

This campaign, which aroused so much indignation among its beneficiaries in the beginning and so much enthusiasm in the end, was due to the propaganda and persistence of Charles Wardell Stiles, a zoologist in the Public Health Service, who in the early 1900's had uncovered the worm at the core of Southern backwardness. Having read in Hirsch's famous *Handbuch* of the prevalence of hookworm anemia in the West Indies, Stiles wondered whether it might not be present in the southern part of the U. S. The parasite had been reported once or twice as a curiosity in such unlikely places as St. Louis (1893) and Buffalo (1897), and in 1901 Dr. Allen J. Smith of Galveston sent him a few hookworms obtained from his medical students at the University of Texas. Stiles however found no specimens from man in the collections of the National Museum, Army Medical Museum or Bureau of Animal Industry. He suspected that the hookworm, as a cause of anemia, was being overlooked and he lectured on the subject at Johns Hopkins, but he says that his good friend Dr. William Osler took him roundly to task and maintained that a disease which could be so easily recognized could not have escaped detection if it were present. Stiles, however, persisted, and obtained permission from Surgeon-General Wyman of the Public Health Service to make a reconnaissance on his own account. He immediately set up a clinic in Columbia, S. C., and made excursions into Georgia and Florida. In a week he had the confirmation of his suspicion; he found to his excitement, that every anemic, listless, pot-bellied child he examined was a victim of the hookworm. He had discovered the cause of the commonest disease in the Southern States.

Stiles reported this on December 4, 1902, to the First Panamerican Sanitary Conference, which had met in Washington to organize an international health office for the Americas, later to be called the Pan American Sanitary Bureau. By a curious circumstance, no speech he ever made caused such commotion or was fraught with such long-term consequences as this one. Stiles said that the so-called "poor white trash" in our South were not constitutionally lazy; their energy was being sapped by hookworms. A reporter from the New York Sun embroidered this in humorous vein and sent in the story that a doctor in the South had discovered the germ of laziness; loafing was just a disease. This diverted his New York office, and the Sun of December 5 headlined the discovery. The ridiculous idea was caught up by newspaper rhymesters and cartoonists, and swept the country. Stiles thought it a fortunate episode. "This reporter," he wrote afterwards, "contributed an exceedingly valuable piece of work in disseminating knowledge concerning hookworm disease. . . . It would have taken scientific authors years of hard work to draw as much attention to this subject."

Actually, the five-year campaign of the Rockefeller Sanitary Commission did not have much immediate effect on the hookworm. The task was not one which could be carried through to any conclusion by a six-weeks' campaign, which was all the time that could be devoted to any one county. To get rid of the hookworm, the standards of living and of education would have to be permanently raised. But it had an enormous effect upon the South; the Rockefeller Sanitary Commission had revealed the magnitude of the health problem, its social repercussions on the community, and the feasibility of its solution. To the Trustees of the newly-created RF, Rose's success in mobilizing the health authorities of eleven Southern States in a concerted drive against a common ill seemed capable of unlimited application. Frederick T. Gates, one of the chief architects of the Foundation and Mr. Rockefeller's personal adviser, determined to organize "a new and world-wide agency to attack hookworm and other curable and preventable diseases, and to promote universal health." That was the origin of the International Health Commission.

Although the word "eradication" had been

rashly inserted into the title of the Rockefeller Sanitary Commission, neither Rose nor Stiles thought it possible within any given time. Even before the Commission had begun its campaign, Rose wrote, "The eradication of the disease is a work which no outside agency could do for a people if it would, and one which no outside agency should do if it could." Nevertheless Rose had earnestly desired at least one complete demonstration, even on a small scale—that somewhere cure and prevention might reach 100 per cent of the inhabitants and serve as a model of perfection after the Commission had withdrawn. In September, 1913, an attempt at eradication was begun on Knotts Island, a remote fishing village on the North Carolina coast which had little contact with the rest of the world. The plan was to treat and sanitate until the job was done. Ninety-four of the 567 inhabitants had hookworms and 90 were eventually declared to be cured after many courses of thymol, a disagreeable and relatively ineffective poison which had to be given repeatedly, and administered to children by bribery or coercion. The other four stubborn old people would have nothing to do with the business. The sanitation campaign was, as usual, less successful. No inoffensive solution of the rural latrine problem had been invented by the doctors or engineers except expensive septic tank installations, and Knotts Island folk went on using "nature's way" in immutable disregard of the calamity-howlers. Attempts to persuade the recalcitrants soon ran into the law of diminishing returns and Rose was finally convinced that perfection was not worth the price. He accepted defeat gracefully for his main purpose had been accomplished, to make hookworm prevention an essential part of public health work in the South. As we learned from a paper presented at this meeting, the hookworm still prospers in many localities, and I doubt whether it has ever disappeared from any area in which it has established itself.

But while Rose was organizing the IHC for a general attack on the hookworm, his attention was drawn in a compelling way to two other diseases, both of first importance as obstacles to human progress, and each peculiarly vulnerable, according to the highest authorities, to a well-directed attack, although none unfortunately had ever been launched. These old, highly destructive enemies were malaria and yellow fever. Passing through London early in 1914 on his way around the world to scout for projects for the IHC, Rose met Sir Ronald Ross, who had received the highest honors including the Nobel Prize for discovering the mosquito transmission of malaria, but who had been in a state of chronic indignation ever since because the knowledge had not been applied on any large scale to control the disease. Gorgas had waged war on the *Anopheles* of the Canal Zone, but at a cost prohibitive to the ordinary community, so that his methods had not been copied even in the U. S. Ross's only enthusiastic and competent disciple had been Malcolm Watson in Malaya, who had persuaded the rubber planters to finance some experiments in *Anopheles* control by drainage. Ross was becoming exceedingly unhappy over the unprogressive attitude of malariologists in general when suddenly here was Rose delivered into his hands, with what was reputed to be unlimited financial backing, and actually looking for a disease to attack. Ross was a very articulate person and Rose was impressed with his grim picture of malaria as a world problem and his exposition of the practicable measures by which it could be controlled. When Rose got to Malaya he took time out from his hookworm observations to visit Malcolm Watson, and came away convinced that the disease was one of the utmost gravity and that much could be done to prevent it. The criteria which he thought should govern the selection of a disease suitable for attack by the IHC were that it should be of global importance, that little was being done about it, and that it could be prevented at a reasonable cost. Malaria satisfied the first two conditions; the question remained, as he wrote in his report, "whether the various known measures, such as quinine treatment, screening, and drainage operations, can be effectively employed . . . at a cost which will not be prohibitive." Rose set to work at once to find out.

Always anxious to involve government at all levels in his projects, Rose asked the U. S. Public Health Service (PHS), which had been gathering data on malaria since 1912 but had no money, whether it would not like to invite the IHC to collaborate with it and the state and local authorities in some experiments in a few selected localities to find out what it would cost to protect a rural community against malaria. Under the direction of PHS officers, chiefly engineers, and at the expense of the IHC, these experiments were begun in 1916 in several Arkansas and

Mississippi villages, and succeeded beyond expectation. Malaria was greatly reduced by one or a combination of measures at reasonable cost and the villages were so pleased that they continued the work at their own expense. During World War I the same methods were used to protect Army encampments and key industrial centers from malaria, and by 1922 the anti-anopheline drive had been extended to 163 counties in 10 states with very satisfactory results, even though no major drainage operations were attempted. Rose was pleased. He had prudently sent a young engineer to Nicaragua in 1920 to see whether the measures, so successful in the Southern States, were adaptable to tropical conditions, and finding that they were, he set up a training station in Leesburg, Georgia, under Dr. Samuel T. Darling to instruct his young "malariologists" in the techniques of making a malaria survey and of applying the standard methods of obstructing the transmission of the infection. By 1922 he felt secure enough to accept invitations from Brazil and Italy to collaborate in solving malaria problems which had not yielded to the classical method of quinine prophylaxis and cure, and soon he had made similar agreements with malarious countries all over the world.

As long as we were fighting the hookworm, it took no great amount of training or epidemiological insight on the part of the staff to circumvent the parasite with its simple mode of spread; it was human nature, not the hookworm, that defeated us. Malaria proved to be a different sort of problem altogether, as I began to suspect when I was transferred to Brazil. Our principal scout in the foreign field was Dr. Mark Boyd, who was sent down to me to survey the malaria situation in the State of Rio de Janeiro. When Boyd, accompanied by the Brazilian entomologist Antonio Peryassú, disappeared into the marshes of Magé, there were only three recognized *Anopheles* in the whole region, but when they emerged months later, no one knew how many there were. They had begun that process of multiplication and division of species which has enriched entomological nomenclature and frustrated the malariologist ever since. For no one was able to tell the variants from the true species, and specimens submitted to more than one systematist would come back with more than one name, so that we cannot say even now how many species of *Anopheles* there are. The aberrant forms were all given names, usually those of persons, like *rooti*, *evansi*, *strodei*, *oswaldoi* etc., which was considered to be an honor to the eponymous individuals. Dr. Peryassú, in a friendly gesture of hospitality and homage, created a new species, *Anopheles rockefelleri*, no specimen of which has ever been captured. What was most confusing was that the same mosquito would appear to be a notable vector of malaria in one area but quite inoffensive in another, a phenomenon which was to puzzle us later in Europe. In spite of all this, the tactics of mosquito control remained the same as in Arkansas: put the water in motion if you can't get rid of it, and *Anopheles* breeding will stop. This "secret" was our main weapon against malaria.

The thing that had made Ronald Ross indignant and had surprised Rose was that the European malariologists did not seem to know this. Rose determined to find out why, and to this end I was rapidly converted in a few weeks at Leesburg from a helminthologist to a malariologist and sent to Italy. My qualifications were that I knew my anophelines through association with Boyd, and my malaria by personally contracting a *vivax* infection in Leesburg and a *falciparum* infection in Panama. I had thus been afforded a splendid opportunity to study at first hand Dr. Bass's standard quinine treatment in various formulations and dosages, though I never got to like it. Dr. William James of the Canal Zone used to double and triple it in obstinate cases like mine and add calomel.

I think I was supposed, when I got to Italy in 1924, to talk rather severely to Grassi, Marchiafava, Bastianelli and other renowned malariologists, and show them lantern slides of the work in Arkansas. What I did of course was to keep my mouth shut and sit at the feet of the masters. What I learned was that the vector anophelines of the Mediterranean countries were more versatile than those of Arkansas—they could fly farther, they were more prone to bite human beings, and would breed in almost any kind of water—brackish or sweet, standing or moving—so that the great drainage canals themselves were a prolific source of mosquitoes. No wonder the Italians had failed to adopt the simple American measures. Their *Anopheles* were more dangerous by far than ours, and much more difficult to reduce in number below the threshold density required to maintain malaria transmission. Considering how best to spend their limited funds, the Italians had fallen back on

intensive quinine therapy, a time-honored resource with a history of 300 years of continual defeat. There were good years, as always in the malaria cycle, which they attributed to the quinine program, and there were bad years which they attributed to acts of God, but the fact was that the death rate from malaria in Italy in 1924 was higher than it had been a decade earlier. The outlook was therefore pretty discouraging to a fellow with my limited experience. There were evidently no easy victories to be won either over the *Anopheles* or over the plasmodia, and I hoped I could make the people in our New York office understand this.

However, I had another weapon up my sleeve in Paris green, which Dr. Marshall A. Barber had just discovered to be an effective anopheline larvicide. I had brought a small supply with me to Italy and tried it out on the quiet in Sardinia. Barber had experimented with it on a small scale at his laboratory in Louisiana, but this was the first time it had been used in actual combat. It worked splendidly on the Italian larvae and was an enormous improvement over anything that had ever been tried before against anopheline mosquitoes. We had no doubt from the first that it would put quinine out of the running as an antimalaria measure.

This was not immediately conceded by some of the European malariologists who were looked up to as authorities. The Second Report of the Malaria Commission of the League of Nations, appearing in 1927, still called for more intensive quininization, and contained the extraordinary statement (and how Ross must have gnashed his teeth!) that the knowledge of the mosquito transmission of malaria had, contrary to all expectation, not helped us in the struggle against the disease, and may in fact have led us away from the right path. The reported success of antimosquito measures in our South was considered a typical American exaggeration if not pure invention, and a commission was sent to the U. S. in 1927 to investigate the matter. It reported that no conclusions could in fact be drawn from the American experience, since malaria was obviously dying out for social and economic reasons unconnected with any specific measures taken against it. Nor was Col. James, the medical adviser to the Colonial Office, impressed by our little show in Sardinia; he wanted a more permanent solution, and thought of malaria as a social disease, perpetuated by bad housing, malnutrition and inadequate medical care, and believed it would tend to disappear spontaneously if the standard of living could be significantly improved. Prof. Marchoux, the leading French malariologist, held that malaria was a disease of backward peoples and would gradually be eliminated as civilization advanced. (He politely refrained from pointing out that France had the least malaria and America the most, of the so-called advanced nations.)

I thought it would be instructive to stage a competition between quinine and Paris green. My Italian colleague, Prof. Missiroli, and I chose two neighboring small villages in Sardinia, which was the most malarious region of Italy, and instituted a treatment campaign in one and antilarva measures in the other, providing equal budgets for the two experiments. The first town had about 1,000 inhabitants and, employing a doctor and a nurse, we succeeded in treating virtually every case of malaria or of parasitemia as soon as it was detected, and we continued this therapeutic campaign for three years. The supply of quinine was unlimited and it was pressed upon the patients in quantities unnecessarily large. In fact, the average consumption per case treated was over 31 grams, three times the amount generally considered ample. It reminded me of old times in Panama. Nevertheless, the amount of malaria actually increased over the three years; the parasite rate went up from 22 to 36 per cent and the spleen rate from 75 to 94 per cent. In the last year we treated 915 individuals, almost everybody in town. The quinine did a great deal of good; it relieved suffering and reduced the death rate, but there was no evidence that it had suppressed endemic malaria in the slightest degree. In contrast, malaria diminished rapidly in the village protected from mosquitoes, and before long there developed an emigration of anemic children from the first town to the second during the worst of the summer months. The Sardinian demonstration put our modest Malaria Experiment Station on the map, nationally and internationally. I was appointed Vice President of the Malaria Commission of the League of Nations, a purely ornamental job if there ever was one, but indicating their change in attitude, and Prof. Missiroli was at last able to obtain the ear of his government, whose authority was then concentrated in the person of Mussolini. It transformed the official malaria-control measures

in Italy, and the use of Paris green spread around the world. The attack on the mosquito now had a decided edge over the attack on the parasite, which it has never lost.

It was Paris green which Drs. Soper and Wilson of the IHD used in Brazil to eradicate *Anopheles gambiae* in 1939–40. This led to another mutation in our thinking about malaria. Long before that time, eradication of malaria had been discussed, but without conviction. Dr. Frederick L. Hoffman, statistician for the Prudential Life Insurance Co. procured in 1916 the formation of the National Malaria Committee as a catalyzing agent for the eradication of malaria from the U. S. and possibly from the Western Hemisphere. It was estimated at that time that more than a million cases and 15,000 deaths occurred annually in the U. S. due to malaria, causing an economic loss which ran into the hundreds of millions of dollars. Virtually nothing was being done about this, and in 1917 Dr. Hoffman read a paper before the Committee entitled "A plea and a plan for the eradication of malaria." He was soon led to the conclusion that this would entail the eradication of the malaria vectors, for he said in 1918, "Any effort must fail to rid the community of malaria if the *Anopheles* mosquitoes are not entirely done away with." That seemed an extravagant proposal in the days of quinine and minor drainage, and the nation was not stimulated to action until Soper's and Wilson's spectacular feat in Brazil. Here was an achievement, accomplished with the tools we all had, which aroused first this country and then the world to revolt against paying annual tribute in lives and fortune to the brutality and exactions of malaria. Dr. Wilbur A. Sawyer, then Director of the IHD, a man of cool and balanced judgment not apt to be carried away by a wave of over-optimistic enthusiasm, became an advocate of mosquito eradication as the safest and most economical way of getting rid of malaria. "The success beyond all predictions," he wrote, "of the major strategy in the extermination of *A. gambiae* in Brazil has fired the imagination of malariologists everywhere." and Dr. L. L. Williams, Jr., began to elaborate the National Malaria Eradication Program which was put into effect by Congress in 1947, and which in turn influenced the WHO to enter upon its present global effort. So in malaria as in hookworm, the IHC set in motion a chain of events which we can describe, in Rose's words, as "bound up in a sure sequence."

The *gambiae* campaign in Brazil had a curious sequel which caused consternation for a brief period throughout South America, but which as far as I know, has never been reported. The last adult *gambiae* was caught in Brazil on November 9, 1940, and continued surveillance for almost two more years failed to turn up a single larva or adult. It was practically certain that every last *gambiae* had died.

But on September 5, 1945, I received a letter at the regional office of the IHD in Buenos Aires from the Rio de Janeiro office calling my urgent attention to an article in the Journal of the Museum of Natural History of Asunción, Paraguay, by a Russian entomologist named Podtiagin, the Curator of the Museum. Podtiagin stated that among some mosquito larvae collected at Colonia Independencia during a very serious epidemic of malaria, he had identified larvae of *A. gambiae*. Podtiagin had worked in Africa and knew the species well. The letter said that he had sent an adult specimen bred out from one of the larvae to the Oswaldo Cruz Institute in Rio, where it had been confirmed by Nelson Cerqueira as undoubtedly *A. gambiae*. Dr. René Rachou of the Brazilian Malaria Service had gone at once to Asunción and I lost no time in getting the news to Dr. Carlos Alvarado who was Director of the Malaria Service of Northern Argentina. Both Brazil and Argentina sent commissions immediately to examine the entire length of their frontiers with Paraguay, but no *gambiae* were found. A joint Brazilian and Argentine committee was then sent to Asunción to interview Podtiagin and review the evidence. Podtiagin suggested at least four routes by which *gambiae* might have entered Paraguay. A plane from North-East Brazil during the *gambiae* invasion might easily have continued its flight to Asunción. The Botanic Garden of Asunción had recently received jars of tropical plants from Africa which might have contained larvae. During World War II a visiting entomologist named Jörgensen, who called himself a Dane but who Podtiagin suspected might have been a German, had been studying the possibilities of "adapting exotic mosquitoes to Paraguay." He died and his widow sold his collections to Podtiagin who found *gambiae* among his live larvae. But in Podtiagin's opinion the most likely means of transport of live *gambiae* from Africa to Paraguay was in one or both of the two gunboats which Paraguay had bought in Italy just before the war and which had

stopped to take on water ballast in Senegal. A Dr. Alexander Ladkovsky, contracted by the Paraguayan government, had boarded one of these gunboats in Algiers and later, in mid-Atlantic, had caught a *gambiae* mosquito on the boat. He also found larvae in the bilge and wrote an article entitled "Mosquitoes of Paraguay" for the Revue de l'Université de Nancy (Vol. I, pp. 68–85, 1938) in which he mentioned this fact.

Podtiagin thought that *gambiae* was rather widespread in Paraguay. Dr. Schenone, a well-known Paraguayan physician, was bitten by a strange-looking mosquito in the Park in Asunción; he caught it and took it to Podtiagin, who recognized it as *gambiae*. But Schenone, a bit skeptical, sent another specimen to his brother who was Military Attaché in Paris asking him to have it classified at the Pasteur Institute. He received a report signed J. Bedoc identifying it as *A. gambiae*. In the meantime an American named Schneider, who collected for North American museums, had sent Podtiagin some adult mosquitoes from the north of Paraguay, some of which were *gambiae*.

Podtiagin, however, had no *gambiae* material to show the Joint Committee, since it had all been destroyed or lost in various ways except the one mosquito sent to the Oswaldo Cruz Institute. The various witnesses too were unavailable. Dr. Schenone had died and Schneider had returned to the U. S. There had been several violent deaths among the group which struck a rather sinister note; Jörgensen was found dead in the jungle with his head bashed in, and Ladkovsky had been assassinated.

The Joint Committee soon found that Podtiagin was not much of an entomologist, but was an expert at intrigue. He spread stories about the Argentines among the Brazilians and about the Brazilians among the Argentines, and set the Paraguayans against them both, with such success that the Joint Committee almost broke up, and Dr. Barbero, President of the Sociedad Científica del Paraguay (which ran the Museum) refused to give the Committee any information. The Committee then got busy running down the secondary clues in this singular affair. Dr. Schenone's brother, the Military Attaché, was traced to Buenos Aires, and his testimony was a shock to the Committee; he had never heard of *A. gambiae*, his brother had not sent a mosquito to him in Paris, and he had never set foot in the Pasteur Institute. The Captain of the gunboat was found and he said he had not picked up anybody named Ladkovsky in Algiers, nor had he taken on water in Senegal. In any case, both gunboats had been laid up for weeks in dry-dock in Buenos Aires to have their bottoms scraped, and could not have transported any mosquitoes to Paraguay. The Botanic Garden had received only dried plants from Africa. What then of the specimen sent by Podtiagin to the Oswaldo Cruz Institute and identified as *gambiae*? Dr. Rachou remembered that Dr. Cesar Pinto of the Brazilian Malaria Service had distributed specimens of *gambiae* to all the South American countries to help them identify any possible invaders from Brazil. He rushed to the Museum, but this specimen could not be found; it had undoubtedly been sent to Rio. The entire business in all its impressive detail had been a carefully prepared hoax. But when the Committee in a body went to charge Podtiagin with fraud, he could not be found; he had eradicated himself from Paraguay. And why did he indulge in this elaborate deception? I think he planned to put himself at the head of a campaign to exterminate a nonexistent *Anopheles gambiae* and to go down in history as the Soper of Paraguay.

I am afraid I have dwelt overlong on malaria, because this is a story I know at first hand. But Rose also selected a third disease against which to wage a war of extermination. While he was in Singapore in 1914, after his visit to Malcolm Watson, he learned that there was apprehension in India and the Far East over the opening of the Panama Canal, which was to take place in August of that year. It was feared that the shortening of the sea route from the Caribbean and Brazilian seed-beds of yellow fever would place the entire Orient at risk, where *Aedes aegypti* was prevalent in many areas. Col. James suggested establishing a quarantine station in some selected port of the Far East such as Hong Kong or Singapore, where all ships from the Canal would be rounded up for inspection before being allowed to proceed to their destinations. Rose knew that the person to see about this was Gen. William C. Gorgas who had just left the Canal Zone to become Surgeon General of the Army. Immediately upon his return Rose got in touch with Gorgas, a consultation which had momentous consequences, for it marked the inception of the largest project which the IHD ever undertook. To Gorgas, the British alarm must have seemed nothing less than providential, if it should lead to a great campaign

throughout the Western Hemisphere, a thing which he had dreamed of but had not thought it possible to realize. He was convinced that yellow fever could remain endemic only in a very few large seaports—not more than five or six perhaps in the American tropics—where babies and immigrants would be numerous enough to provide a constant supply of new fuel. Whenever yellow fever broke out elsewhere in epidemic form, it was a blaze which always rapidly burned itself out. Gorgas's experience had been that the disease, though occasionally able to spread for thousands of miles along routes of travel, could not long withstand a well-directed attack on its "key centers." Thus the campaigns in Havana, Panama and New Orleans had freed the entire Caribbean area from yellow fever. "The IHC," he told Rose, "could not undertake a better piece of work than this. . . . The eradication [of yellow fever] would command the attention and gratitude of the world—and the thing can be done!" In fact, the IHC was the only agency which could wage such an international campaign, for the Pan American Sanitary Bureau at that epoch was an intelligence office without authority or funds. After three weeks of reflection, the Commission resolved to undertake the task. Rose thought it might take at least two years, but Henry Rose Carter, the distinguished epidemiologist of the Public Health Service, wise and cautious as he was, wrote to Rose: "I think you have rather exaggerated the difficulty of getting rid of yellow fever when you put two years as the time required. We know just what to do. There is no uncertainty in it." It had only taken Dr. Joseph H. White six weeks to end transmission for all time in New Orleans in 1905. The prudent Rose however set up the project for a five-year period. The dangers of unconditional prophecy are well illustrated by the fact that it took the IHD 34 years to complete its yellow-fever program; 76 staff members were associated with it at one time or another, and it cost the Rockefeller Foundation almost 14 million dollars.

Gen. Gorgas made a survey of the yellow fever situation in South America in 1916, and while he was in Bogotá, Colombia, with his Commission, inquiring in vain about outbreaks of acute disease in the interior which might be yellow fever, a telegram was received by the National Health authorities reporting an epidemic of yellow fever in the town of Muzo, an isolated spot near some emerald mines, 2,700 feet above the sea and 100 miles northeast of Bogotá. This was very pat indeed, and the General and three of his companions got on horses and rode for several days up the mountains to Muzo. When they arrived they found no very sick people and no cases that suggested yellow fever; more than that, they found no adults or larvae of "*Stegomyia*" (the old name of *Aedes aegypti*), which clinched the case against there having been any yellow fever there. The Committee's report went on to say: "The Alcalde of the town frankly admitted that his telegram had been without foundation; that he had received a number of communications from high officials in Bogotá asking about yellow fever; that for some reason his superiors wished to have the disease in Muzo; and that it was his duty as a subordinate appointed by Government to meet the demand of his superiors; if the Government wanted yellow fever in Muzo, the Alcalde must furnish it. The Commission believes that this mining region may be dismissed from the area requiring consideration." However, jungle yellow fever was surely endemic in Muzo at that very time as later outbreaks proved. Probably the epidemic was not purely an invention of the Alcalde, but was over before the General arrived. As it was, the possibility of yellow fever in the absence of *Stegomyia* was not entertained, and the Commission came to the conclusion that in all South America the only endemic center at the time was Guayaquil.

Dr. M. E. Connor of the IHC cleaned yellow fever out of Guayaquil and all of Ecuador in six months. Unfortunately, that did not ring the knell of yellow fever in the Hemisphere. He had hardly recorded the last case in Guayaquil in May, 1918, when an epidemic was reported from Guatemala, and soon it was cropping up all over the map. In 1919 there were outbreaks in Peru, Brazil, Honduras, Salvador and Mexico, followed within a year or two by epidemics in Nicaragua and in Colombia without known antecedent cases and at points completely isolated from any possible sources of infection. Thus perished the key-center concept, but what was wrong with it was not known for many years, until finally in 1932 a rural epidemic, materializing out of nowhere, occurred in the Valle do Chanaan, in Brazil, in the complete absence of the *Aedes aegypti* mosquito, and it was discovered that yellow fever was not only a disease of man but of monkeys too, spread by forest mosquitoes not vulnerable to any conceivable method of attack.

Thus the hope of banishing yellow fever from

the earth was sunk by jungle yellow fever, but much was salvaged when that ship went down. By 1937, a vaccine had been perfected which protected people from infection by jungle mosquitoes, and a blood test for immunity—the mouse-protection test—had been evolved in 1930 which revealed past infections with the virus in man or monkey. This made it possible to map the endemic areas of yellow fever and even localize previous epidemics in time and place if they had occurred within the lifetime of the people examined, thus giving intelligence of the enemy. A survey of the world for yellow fever immunity in man was at once instituted by Dr. Sawyer in 1931, which revealed a much wider distribution of the virus in South America and Africa than had been suspected. This survey took five years, and since it led the yellow fever staff into the rain forests of both continents and among primitive and even savage peoples, it was attended by hardship and danger. Some of the stories which came out of the jungle have acquired by multiple passage from mouth to mouth happy accessions and incrustations of myth which give them almost a legendary quality.

There is one I prize about Dr. Allen M. Walcott, the dependable troubleshooter of the yellow fever crew. One of his missions took him over the wind-swept Andes of Ecuador by mountain trails into the Amazon jungle between the Pastaza and the Napo rivers, a rugged trip if there ever was one. This is the country of the warlike Jíbaros, the notorious head-hunters of the Upper Amazon, some of whose blood he hoped to obtain for the test, along with that of any of the fabled Amazons he might meet, the beautiful nude warriors on horseback. As his colleagues wrung his hand at his departure and said goodbye, it crossed their minds that one of them, idling some day in a shop in Belém do Pará, might come upon a shrunken head as small as your fist and cry, "Good heavens, that's Walcott!" But Walcott turned up in Iquitos with his head on his shoulders and the blood of the Jíbaros in his bag. I wrote Walcott the other day to try to confirm this story, but he said it was mostly invention—that is, he had made the trip and collected a lot of blood, but had run across only three teen-age Jíbaros, who put up no resistance. He was silent on the subject of the Amazons. His version and mine are at variance only statistically, as you see, and I do not regard the difference as significant.

I will close on this note of modest claims and successful accomplishment, which was characteristic of Wickliffe Rose and in some measure, I like to think, descended to his staff, who admired and were loyal to him. It is ironical, as I look back on the three diseases selected for demonstrations of control, that hookworm disease, chosen for its simplicity as target for the first eradication campaign of them all, has turned out through defects of the human temperament to be the most intractable, and that yellow fever, which Dr. Carter gave two years to live, now appears to be immortal. But malaria, protean and tenacious, the endlessly adaptable and resourceful foe, may well be on the way to extinction. I have sketched for you the earliest flowering of international public health, the tentative efforts of a private organization to stir oppressed peoples to work for their own salvation. To paraphrase the poet, all this I saw and no small part of it I was. The International Health Division, its task as a catalyst accomplished, is no more; but its soul goes marching on.

STUDIES ON TRACHOMA*

1. Isolation and Identification of Strains of Elementary Bodies from Saudi Arabia and Egypt

E. S. MURRAY, S. D. BELL, Jr., A. T. HANNA, R. L. NICHOLS, and J. C. SNYDER

Trachoma and related diseases of the eye afflict millions of people and cause serious impairment of vision in many parts of the world.[1] Most of the countries of the Middle East have been severely involved. In 1954 a study was begun in Saudi Arabia to establish, if possible, the etiologic agents of the common diseases of the external eye, including trachoma, the cause of which has been much disputed in the past. The investigations consisted of clinical, epidemiological and laboratory observations, and recently have been extended to Egypt and other countries of the Mediterranean region. Attempts to recover etiologic agents from conjunctival scrapings of persons with clinical trachoma have revealed a range of potential pathogens,[2] including bacteria, adenoviruses[3,4,5] and elementary bodies.[6] It is the purpose of this series of papers to record our observations and experiments with the viruses of the latter category; the first paper presents the data pertaining to the properties of several strains of elementary bodies isolated from Saudi Arabia and Egypt.

In 1907, Halberstäedter and von Prowazek[7] described small coccobacillary forms occurring intracellularly which they considered to be the etiologic agent of trachoma. They observed the microorganisms in Giemsa-stained smears of conjunctival cells from patients with clinical trachoma. In the succeeding 50 years, their observations have been confirmed by many workers (literature reviewed by Nataf[8] and Thygeson).[1] The small coccoid forms described by Halberstäedter and von Prowazek were termed elementary bodies, while the larger forms were called initial bodies; the intracellular aggregates of elementary bodies were called inclusion bodies.

Many attempts have been made to culture trachoma viruses.[9] Viruses were successfully transferred by inoculation of the conjunctivae of the higher anthropoid apes, but lower animals were resistant to infection. In 1944 Macchiavello reported the growth of a virus in the yolk sac of embryonated eggs;[10] his strain was lost. In recent years there have been intensive trials by several investigators to cultivate trachoma viruses in cell culture, but without success.

In 1957, Tang, Chang, Huang, and Wang reported the isolation of strains of elementary bodies from conjunctival cells obtained from patients with clinical trachoma.[11] They inoculated specimens of infected conjunctival cells into chick embryos using a modification of the technic of Cox[12] and Macchiavello.[10] The findings of Tang and his colleagues were confirmed and extended by Collier and Sowa who isolated strains from trachoma patients in Gambia, West Africa.[13] Collier, Duke-Elder, and Jones inoculated the Gambian strain into the eye of a blind human volunteer who developed typical microscopic and clinical findings of classical trachoma.[14]

Giroud, Renoux, and Nataf described the isolation of elementary bodies by intranasal inoculation of mice, and they reported success in establishing a strain on the chorio-allantoic membrane of chick embryos.[15] They reported that their strains, after several passages in mice or eggs, induced clinical trachoma in two human subjects. Murray, Snyder and Bell isolated a strain of elementary bodies from Saudi Arabia in the yolk sac membrane of chick embryos; they showed that the strain was toxic for mice and induced inclusion bodies in a chimpanzee.[6]

METHODS

The clinical and epidemiological studies were conducted principally among residents of Al Hasa and Qatif, two large oases of eastern Saudi Arabia. In a few instances material was obtained from patients attending eye clinics, either at the Dhahran Health Center, or in the villages. The Egyptian strains were obtained from patients of the outpatient clinic of the Giza Memorial Ophthalmological Institute in Cairo.

* From the Department of Microbiology, Harvard School of Public Health, Boston 15, Massachusetts, and the Aramco Trachoma Research Program, Medical Department, Arabian American Oil Company, Dhahran, Saudi Arabia.

Clinical and Epidemiological Features. A case of "pure" trachoma is a rarity in Saudi Arabia or in Egypt. Under ordinary village conditions an infant becomes infected with one or more pathogenic agents usually during the first few months of life. The resulting conjunctivitides produce chronic mucopurulent discharges and papillary hypertrophy, tending to mask the symptoms and signs of "pure" trachoma. Under these conditions it is difficult to establish accurately the time of onset of trachoma on the basis of clinical findings. Insofar as possible, however, diagnoses were based on the criteria as stated by the Expert Committee of the World Health Organization[16,17] and as presented by Thygeson.[18] Attempts at isolation of elementary bodies were made using specimens from 44 children whose conjunctival scrapings met the morphologic criteria in a subsequent paragraph, and whose clinical findings were those of trachoma II or III, with a single exception as noted in Table 1. The age of the children ranged from 4 months to 7 years. Thirty-nine were residents of eastern Saudi Arabia and five lived in Egypt. Twenty-four individuals were among the large groups which were encountered in survey type visits, whereas twenty were children brought to a clinic because of their parents' concern over their eyes.

Conjunctival Specimens. Material scraped from the surface of the palpebral conjunctiva was subjected to three procedures: morphologic examination by Giemsa stain, culture for bacteria and pleuropneumonia-like organisms, and preservation at −60°C for subsequent virus isolation attempts. The technic of obtaining conjunctival specimens consisted of scraping the tarsal surface of the everted upper eyelid with a sterilized steel instrument. The instrument was bent at an angle of 60° for the distal 10 mm; the outside edge of the bent portion was intermediate in sharpness between an ordinary table knife and a surgical scapel. The scraping stroke was very light, and was intended to obtain only the superficial conjunctival cells on the outside edge of the instrument. Bleeding was not produced unless the conjunctivae were severely inflamed. No anesthetic was used.

The specimen of conjunctival cells adhering to the instrument was processed as quickly as possible; about three quarters of the specimen was scraped off onto a small glass cover slip, approximately 4 mm square, which was dropped at once into a 4 ml screw cap specimen vial containing 0.2 ml of sterile preserving fluid. The remainder of the specimen was then smeared on a glass microscope slide for subsequent staining with Giemsa and examination for inclusion bodies. A culture for bacteria was taken with a platinum bacteriological loop by a light sweep across the lower palpebral conjunctiva. Blood agar and/or chocolate agar plates were used as culture media. In a few instances, as noted in the table, specimens were inoculated into chick embryos directly, without having been frozen for storage.

Preservation of Specimens. Four different fluids were used in the vials in which the conjunctival specimens were placed at the time of collection: (I) "sucrose PG",[19] a medium containing sucrose and glutamate buffered with phosphate; (II) sucrose PG plus 10% to 20% horse serum; (III) "maintenance solution"[20] plus 10% horse serum; (IV) maintenance solution plus 20% horse serum and 5% human serum. Many of the screw cap vials containing conjunctival scrapings were shipped by air from Saudi Arabia to the laboratory at the Harvard School of Public Health for study. During shipment the vials were packed in ordinary domestic canning tins which had been tightly sealed to prevent access of CO_2 vapor to the specimens. The sealed tins were packed in solid CO_2 during the shipment.

Technic of Egg Inoculation and Isolation of Elementary Bodies. Specimens, either freshly obtained, or those which had been stored for variable periods of time at −60°C in an electric refrigerator (obtained from the Revco Co., Deerfield, Michigan), were mixed with sucrose PG containing enough streptomycin to yield a final concentration of approximately 1,000 units per ml. This mixture was kept at +4°C for 1 to 4 hours, and then inoculated, in amounts varying from 0.5 ml to 0.75 ml, into the yolk sacs of one or two chick embryos which had been incubated at 37°C for 6 to 8 days. Subsequent to inoculation, the eggs were incubated at 35°C and were candled at least once daily thereafter.

In some instances the first passage eggs died in the period from the 6th to the 9th day after inoculation. Embryos which were still alive on the 8th or 9th day after inoculation were sacrificed. Impression smears were made of yolk sacs of dead or sacrificed eggs and stained by the methods of Giemsa and of Macchiavello.[21] If the smears showed no bacteria, the membranes were considered suitable for passage and the

approximate number of individual elementary bodies was recorded as 0—none seen in 30 minutes search of the smear; A—1 to 10 per oil immersion field using 7.5× ocular and 100× objectives; B—10 to 100; and C—more than 100. Although intracellular accumulations of elementary bodies were sometimes seen in yolk sac smears, it was more common to find the individual elementary bodies scattered across the smears. The membranes selected for passage were sometimes stored at −60°C for varying periods, or used at once, depending on the availability of eggs of appropriate age. Passages were made as follows: yolk sacs were mixed with 3 parts of sucrose PG; the mixture was shaken with beads in a screw cap glass vial for several minutes; approximately 0.2 ml to 0.4 ml was then inoculated into the yolk sac of 6 to 8 day old embryos. At least three blind passages were made before abandoning an isolation attempt as unsuccessful.

Microscopic Features of Conjunctival Scrapings. Conjunctival smears were fixed with absolute methyl alcohol, and then stained for 45 minutes with stock Giemsa diluted 1 to 25 in phosphate buffer, pH 7.2. The buffer was kept at +4°C as a concentrated stock solution and it was diluted tenfold in distilled water just before use. After staining, smears were washed for 10 seconds in 95% ethyl alcohol to wash off excess stain. Smears were examined microscopically first under low power and then under oil immersion. A minimum period of 30 minutes of study was carried out before considering a slide as negative.

The sole criterion for considering a slide as positive was the finding of one or more intracellular aggregates of elementary bodies. In most cases the scrapings contained both elementary and initial bodies. Inclusions consisting entirely of initial bodies were not considered as positive since the latter are frequently similar in appearance to the intracytoplasmic aggregates of bacteria sometimes found in bacterial conjunctivitides. Occasionally the elementary bodies appeared singly in the cytoplasm, but the slides were not considered as positive unless aggregates consisted of more than 10 elementary bodies, closely packed in an inclusion. Classically, these inclusions appeared in the cytoplasm in juxtaposition to the nucleus, often forming a half moon or cap which partly encircled it. The elementary bodies stained a variable dark or light pinkish violet while the larger initial bodies stained a deeper dark bluish violet. The positive conjunctival slides were arbitrarily rated as 1, 2, 3, or 4. In a few cases a count was made, recording the number of cells containing inclusions out of the total number of conjunctival cells examined on the slide.

Toxicity Tests. After establishing strains by repeated passages in eggs, those which were very rich in elementary bodies were tested for toxicity, as follows: yolk sacs from embryos *surviving* on the 6th to the 8th day after inoculation were selected. The membranes were mixed thoroughly, either mechanically or by shaking with glass beads, using sucrose PG as the diluent. The suspensions were diluted to 30% yolk sac in sucrose PG and centrifuged at 1,500 rpm for 15 minutes to eliminate gross particles. Young albino Swiss mice were lightly anesthetized with ether and inoculated intravenously with 30%, 20% and 10% suspensions of yolk sac in sucrose PG on the basis of 0.25 ml of yolk sac suspension per 10 g of body weight. The mice recovered promptly from anesthesia and were normally active for at least 55 minutes after inoculation. In an occasional instance a mouse was eliminated from consideration because of abnormal signs in the hour immediately after inoculation or because of failure to recover from the anesthesia. The test was arbitrarily terminated at the end of 24 hours. Deaths attributed to toxin regularly occurred in the interval from 2 to 8 hours after inoculation, and hemorrhagic exudate in the upper small intestine was the characteristic abnormality at autopsy.[6] Differences between some of these strains with respect to active immunization of mice against toxic death have been noted elsewhere.[22]

Complement Fixation Tests. Antigens were prepared from pools of those strains which after several passages in eggs were toxic for mice and which contained in Giemsa stained smears at least 500 elementary bodies per oil immersion field. The antigens were prepared by boiling, using Terzin's modification[23] of Bedson's method.[24] Three or 4 antigen units were used in the CF test. Controls were prepared from normal yolk sac suspensions. Standard positive reference psittacosis sera were supplied through the courtesy of the Rocky Mountain Laboratory of the

U. S. Public Health Service and Dr. Fritz Dekking of the Institute of Tropical Hygiene, Amsterdam, Holland.

Miscellaneous Observations. (a) Pleuropneumonia-like organisms. The presence of these microorganisms was suspected in every strain until negative cultures[25] with appropriate controls had been obtained. (b) Other viruses of the psittacosis-LGV group. Each suspected strain was inoculated into weanling mice by the intracerebral route; the test mice were observed for 3 weeks for signs suggestive of psittacosis or other viruses of the group which are pathogenic for mice. (c) Pathogenicity for human epithelial cells. Eight strains were inoculated into roller tubes containing either conjunctival cells[26] maintained in MS and horse serum,[20] or "HEP" cells,[27] a strain of epithelial-like cells derived from a human carcinoma. The inocula were suspensions of infected yolk sacs at 10 to 30% in sucrose PG. The tubes were observed for at least 3 weeks and transfers to new tubes were made for 3 passages in some instances. On a few occasions highly concentrated suspensions of elementary bodies were also used as inocula. Several of the tissue cultures were subsequently inoculated into eggs as an assay for viable elementary bodies.

RESULTS

Forty-five attempts were made to establish strains by inoculation of chick embryos with conjunctival scrapings, aliquots of which showed typical inclusions. All of the patients had clinical trachoma except one (see SA-5 in Table 1). This 9-month-old patient on repeated examinations had numerous inclusions in her scrapings, but her clinical findings were noted only as "mild conjunctivitis."

Elementary bodies were isolated from 19 of the 45 trials; nine of these strains have been subjected to the various procedures reported above. The elementary bodies of each of the strains had the morphologic properties which are regarded as characteristic of viruses of the psittacosis-lymphogranuloma group. Their appearance in Giemsa-stained smears was identical to the description given by Collier and Sowa.[13] The iodine stain was not applied to any of the yolk sac material. The elementary bodies stained bright reddish pink by Macchiavello's stain. Table 1 presents the data pertaining to the 9 strains which were thoroughly studied.

The data in the table indicate various points of interest. Strains were isolated from several different localities in eastern Saudi Arabia, during the years 1956, 1957, and 1958. Storage of the conjunctival scrapings in sucrose PG for periods up to 20.5 months at −60°C did not destroy the viability of the elementary bodies. Isolation attempts with specimens stored in sucrose PG were more successful than with those stored in the other media; 6 of the 9 strains in Table 1 were initiated from scrapings placed in sucrose PG. (If the incompletely studied isolates are also included, the positives from sucrose PG were 15 out of 16 attempts, whereas there were only 4 positives out of 29 attempts with other media.)

Strains SA-2 and SA-4 were obtained from the same subject, a 3-year-old child whose conjunctivae were scraped on two occasions, 4 weeks apart.

Isolation of strains was successful not only with children in the clinics, (brought because of the parents' concern over their eyes), but also with individuals observed in surveys. (The ratios of positives to total attempts, combining both the 9 completely studied and the 10 other isolates, were: 13/25 for surveys, 6/20 for clinics.)

Properties of Strains in Egg Passages. Table 1 shows data for the first 3 egg passages for each of the 9 strains as well as the total numbers of passages accomplished thus far. One feature has been impressive, namely, the variability among the strains with respect to the attainment in consecutive passages of consistently high concentrations of viable elementary bodies as judged by lethal endpoint titrations in eggs, toxicity for mice, and numbers of elementary bodies in smears. Strains SA-2, 5, 8, and Egypt-2 were regularly more predictable than SA-1, 3, and 6. With all of the strains there has been more difficulty in preparing rich pools than with various rickettsial strains.

Toxicity for Mice. Eight of the 9 strains were toxic for mice when 20 to 30% suspensions of heavily infected yolk sacs in sucrose PG were inoculated intravenously. In the case of strain SA-3, it was very difficult to obtain pools which were rich in elementary bodies and it was necessary to concentrate the material to the equivalent of 400% yolk sac by 2 cycles of centrifugation at 5,000 rpm in order to demonstrate any toxicity for mice. Material derived

TABLE 1

Data pertaining to 9 strains of elementary bodies isolated from Saudi Arabia and Egypt

Strain designation	SA-1	SA-2	SA-4	SA-3	SA-5	SA-6	SA-8	SA-13	Egypt -2
Patient									
Home	Al Omran	Um es Sahich	Um es Sahich	Hazim Ujam	Al Awamieh	Mubarrez	Safwa	Kilabia	Giza
Age	18 mo.	3 yr.	3 yr.	5.5 yr.	9 mo.	5 yr.	5.5 mo.	7 yr.	4 yr.
Sex	F	M	M	F	F	M	F	M	F
Clinic or survey	Survey	Survey	Survey	Survey	Survey	Survey	Survey	Clinic	Clinic
Clinical data									
Right eye	Tr I+P	Tr II+F++P	Tr II+F++P	Tr II++F+P	Mild conjunc-	Tr III	Tr II	Tr II	Tr II+F
Left eye	Tr I+P	Tr II+F++P	Tr II+F++P	Tr II++F+P	tivitis	Tr III	Tr II	Tr II	Tr II+F
Inclusions in conjunctival scrapings (see text):									
Right eye	2	2	1	1	48/600	5/100	15/400	3/1000	9/300
Left eye	1	3	1	1	12/600	10/450	14/300	12/1600	3/150
Date scraping obtained	26 July 57	23 Oct 56	20 Nov 56	20 Nov 56	3 July 58	2 Aug 58	9 Aug 58	19 Mar 58	31 Aug 58
Preserving fluid (see text)	III	II	I	I	I	I	I	II	I
Duration of storage at −60°C before egg inoculation	10 mo.	20.5 mo.	19.5 mo.	19.5 mo.	7 days	None	2 days	7 mo.	None
Egg inoculations	Day of death (D), or sacrifice (S). Number of elementary bodies in smears (0, A, B, C—see footnotes).								
1st passage	8, S, A	8, D, B	9, S, C	9, D, C	7, S, C	9, D, A	8, S, B	8, S, O	6, D, A
2nd passage	8, D, B	7, D, B	5, D, C	6, D, B	5, D, B	7, S, B	8, D, B	7, S, C	6, D, B
3rd passage	5, D, B	5, D, B	6, D, C	11, D, B	5, D, B	7, S, A	6, D, C	4, D, A	8, D, B
Number of egg passages to date, 9/22/59	27	35	6	16	25	9	8	10	16
LD_{50} for eggs[p]	10^6	10^8	10^6	10^6	10^8	q	q	q	q
Toxicity for mice (per cent yolk sac)	25%	20%	25%	r	30%	30%	30%	30%	20%
CF titer of boiled antigen vs. standard positive psittacosis serum	1/160	1/80	>1/160	1/40	1/320	1/10	1/320	1/10	1/80

O—Elementary bodies not observed in 30 minutes' search of smear.
A—1 to 10 elementary bodies per oil immersion field (8× oculars, 100× objectives).
B—10 to 100 elementary bodies per oil immersion field (8× oculars, 100× objectives).
C—More than 100 elementary bodies per oil immersion field (8× oculars, 100× objectives).
p—Maximum number of egg LD_{50} units per gram of yolk sac.
q—Titration not attempted. Dilutions of 10^{-2} were lethal in routine passages.
r—Toxic only when highly concentrated (see text).

from normal yolk sacs processed in the same manner did not kill mice.

Common Group Antigen. Each of the 9 strains was shown to have the heat stable common antigen of the psittacosis-LGV group, in titers ranging from 1/10 to 1/320 when tested against standard positive psittacosis sera. These antigens were prepared without difficulty, a positive result usually being obtained in the first attempt.

Miscellaneous Tests. Each of the strains was shown to be free of pleuropneumonia-like organisms. None was pathogenic for mice by intracerebral inoculation of 10 to 30% yolk sac suspensions. Although a few intracellular aggregates of elementary bodies, very similar to classical inclusions, were occasionally observed in tissue cultures within a few days after exposure to 10 to 20% yolk sac suspensions of the various strains, in no instance was any of these strains established by serial passage in conjunctival or HEP cells maintained in MS and horse serum. None of the eggs died after receiving tissue culture passage material.

Pathogenicity. It was reported previously that strain SA-1 induced conjunctival inflammation in a chimpanzee.[2, 6] Figure 1 is a photograph of the inclusions observed by Giemsa stain in the conjunctival scraping of the chimpanzee on the 2nd day of clinical disease.

During the period when our laboratory staff was working exclusively with egg passages of strains SA-1, 2, 5, and Egypt-2 a severe case of trachoma occurred in the technician who was inoculating eggs and processing the yolk sac membranes. No overt break in technic was recalled, and the infection was presumed to have been caused by airborne droplets of infected yolk sac membranes, probably strain SA-1, a large pool of which was subjected to several procedures by the technician just 3 days prior to the onset of her infection. The details of the case will be reported elsewhere.[28] Figure 2 shows the appearance of Giemsa-stained conjunctival cells scraped from the left eye of the technician on the 4th day of clinical disease. Strains of elementary bodies were isolated from conjunctival

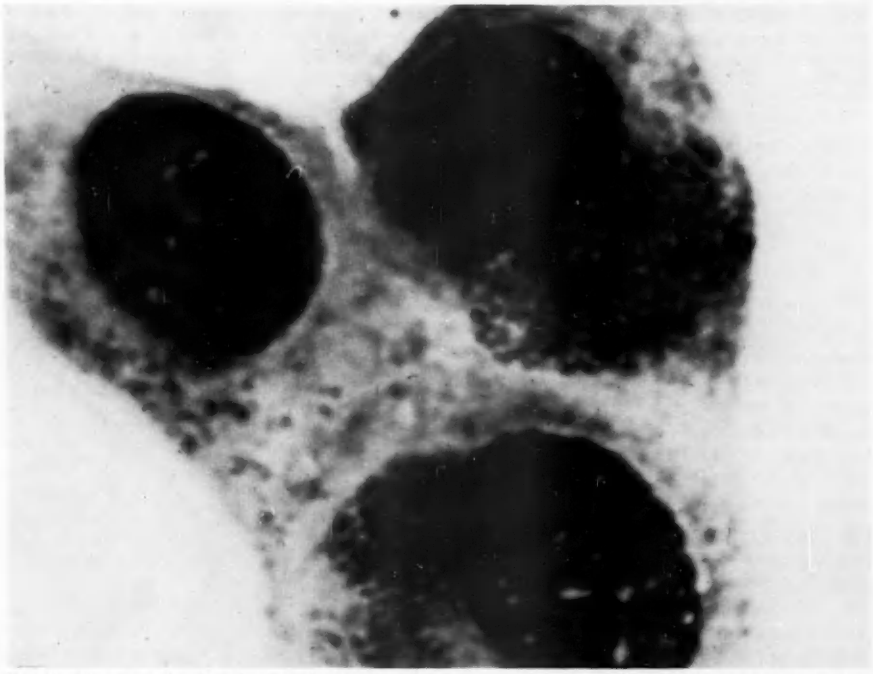


FIG. 1. Photograph of inclusions in Giemsa-stained conjunctival cells of chimpanzee obtained on second day of conjunctival reaction. The chimpanzee was inoculated with 0.05 ml of 10% yolk sac, strain SA-1, second egg passage. (× 2800)

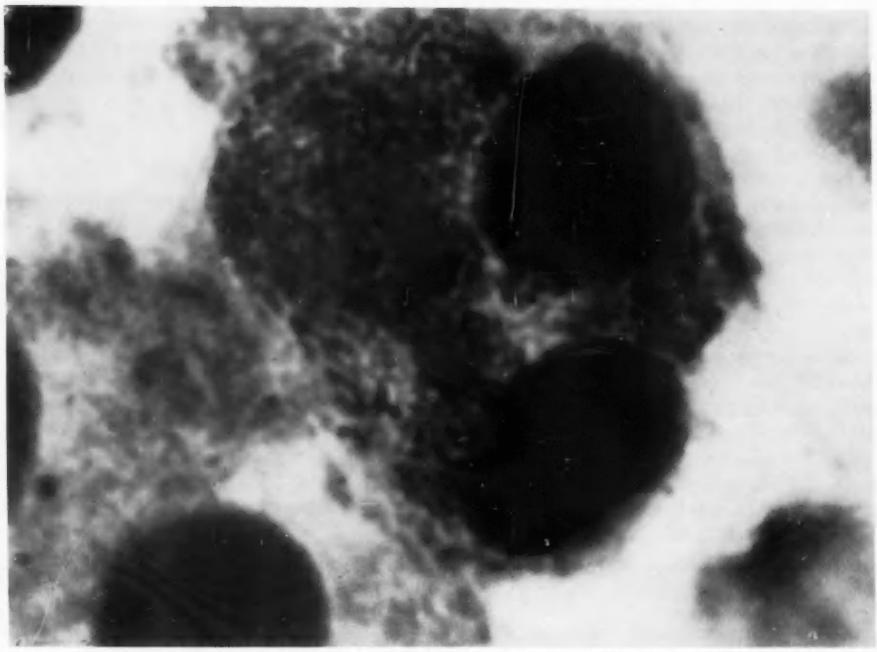


FIG. 2. Photograph of inclusion in Giemsa-stained conjunctival cell scraped from the laboratory technician who contracted clinical trachoma during the period when she was processing concentrated suspensions of elementary bodies. The scraping was made on the 4th day after onset of the infection. (× 2800)

scrapings obtained on the 2nd and 4th days after onset of signs and symptoms. The infection responded to treatment with oral sulfonamides and topical tetracycline.

DISCUSSION

The data reported above constitute a confirmation of the reports by Tang *et al.*[11] and Collier *et al.*[13, 14] of the successful isolation of elementary bodies from trachoma cases using streptomycin and the yolk sac route of inoculation of chick embryos. In our tests successful isolation was obtained in fewer than half the attempts even though the specimens were all inclusion positive. This low rate may be attributable to adverse environmental conditions during storage of the specimens, since one preserving fluid was clearly superior to the three others which were included in the tests. This point is being investigated with particular reference to the influence of freezing and storage on viability.

The morphologic identity of the strains and their content of common group antigen might lead one to assume identity in other properties as well, but it is the conviction of the authors that the rather impressive differences among the strains in respect to regularity of attainment of high titers in eggs probably indicates basic differences rather than chance variations. This view is supported by the experiments on active immunization of mice against toxic challenge.[22]

The question could be raised as to the possibility of revealing agents latent in chick embryos by repeated serial passages. The eggs used in our tests have been obtained from the poultry farm which has supplied our eggs for many years. Repeated checks for latent agents have revealed only occasional pleuropneumonia-like organisms which were ruled out in the experiments here reported.

The failure to obtain unequivocal propagation of the trachoma elementary bodies in cultures of human conjunctival cells is a very challenging observation, the explanation of which, if elucidated, may throw light on the complex relation-

ships of the elementary body parasite to the tissue cells, the nutrients, and other factors which are still to be identified.

The data now available justify the view that the elementary bodies which have been propagated in yolk sac of the chick embryo by Tang *et al.*,[11] Collier *et al.*[13, 14] and as reported above, can induce clinical trachoma in man and that the organisms can again be recovered in laboratory passage. It is not possible, however, to justify an assertion that clinical trachoma is a disease entity with a single etiologic agent. There are several observations which suggest important differences from one region to another, in respect to (a) success in isolation of strains of elementary bodies, and (b) abundance of inclusions in conjunctival scrapings in some regions as opposed to extreme rarity in other regions, despite identity of clinical findings and standard technics of investigation. It is also possible that agents other than viruses of the elementary body group can induce conjunctival reactions which mimic early clinical stages of classical elementary body induced trachoma.[4, 5] Our current studies did not include simultaneous search for adenoviruses and elementary bodies from the same specimen because the quantity of infectious material was so small that we hesitated to divide the specimens. It may be noted, however, that several adenoviruses (types 8 and 15, and Strains 931 and BAR-2) have been isolated from conjunctival scrapings of children who had classical inclusions at the time the specimens were taken for virus isolation attempts.[29]

SUMMARY

This paper reports the isolation of strains of elementary bodies from trachoma cases in Saudi Arabia and Egypt by inoculation of the yolk sacs of chick embryos. Nineteen strains were obtained in 45 attempts using conjunctival scrapings from children who were shown to have typical inclusions at the time of the scraping. Nine of the strains were subjected to a series of tests which showed that they were indistinguishable morphologically from the elementary bodies of the psittacosis-lymphogranuloma group of viruses, that they were not sensitive to streptomycin, that they were free of other viruses and of pleuropneumonia-like organisms, that they were toxic for white mice by the intravenous route, and that they contained the heat stable antigen which is common to the psittacosis-lymphogranuloma group of viruses. Although each strain was capable of multiplication in yolk sac membranes of chick embryos, irregularities of growth were frequently observed, being more characteristic of certain strains, than others.

Viability of the trachoma elementary bodies in conjunctival scrapings persisted at −60°C for many months if the specimens were stored in a medium containing sucrose, glutamate, and phosphate. One of the strains induced conjunctivitis in a chimpanzee whose conjunctival cells showed typical inclusions at the height of the conjunctival reaction. A laboratory worker contracted severe trachoma probably as a consequence of air-borne droplets arising from manipulations of concentrated suspensions of the elementary bodies.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Dr. Robert C. Page and Dr. Richard H. Daggy of the Medical Department of Aramco and to Dr. Ibrahim Ahmed Mohamed of the Giza Ophthalmological Institute for their help in making these studies possible. The authors are grateful to Miss Dorothy McComb, Miss Jane Driscoll, and Miss Barbara Theobald for excellent technical assistance.

REFERENCES

1. Thygeson, Phillips, 1959. Trachoma and Inclusion Conjunctivitis, chapter 34 in *Viral and Rickettsial Infections of Man*. ed. by Rivers and Horsfall, 3d ed., J. B. Lippincott Co., Philadelphia.
2. Snyder, J. C., Page, R. C., Murray, E. S., Daggy, R. H., Bell, S. D., Jr., Nichols, R. L., Haddad, N. A., Hanna, A. T., and McComb, D., 1959. Observations on the etiology of trachoma. *Am. J. Ophth.* (in press).
3. Murray, E. S., Chang, R. S., Bell, S. D., Tarizzo, M. L. and Snyder, J. C., 1957. Agents recovered from acute conjunctivitis cases in Saudi Arabia. *Am. J. Ophth.*, **43:** 32 (Abst.).
4. Bell, S. D., Jr., McComb, D. E., Murray, E. S., Chang, R. S., and Snyder, J. C., 1959. Adenoviruses isolated from Saudi Arabia. 1. Epidemiologic features. *Am. J. Trop. Med. & Hyg.*, **8:** 492–500.
5. Feng, M., Chang, R. S., Smith, T. R., and Snyder, J. C., 1959. Adenoviruses isolated from Saudi Arabia. II. Pathogenicity of certain strains for man. *Am. J. Trop. Med. & Hyg.*, **8:** 501–504.
6. Murray, E. S., Snyder, J. C., and Bell, S. D., Jr., 1958. A note on the toxicity for

white mice and gerbilles of a strain of elementary bodies isolated from a patient with trachoma in eastern Saudi Arabia. *Proc. VI Internat. Cong. Trop. Med.*, Lisbon, Portugal, (in press).

7. Halberstaedter, L., and von Prowazek, S., 1907. Zur Aetiologie des Trachoms. *Deutsche med. Wchnschr.*, **2:** 1285–1287.
8. Nataf, Roger, 1952. *Le Trachome*. Masson et Cie, Paris.
9. Thygeson, Phillips, 1939. Cultivation of trachomatous conjunctival epithelium in vitro. *Arch. Ophth.*, **21:** 229–234.
10. Macchiavello, A., 1944. El virus del trachoma y su cultivo en el saco vitelino del huevo de gallina. *Rev. ecuatoriana de Hig. y Med. Trop.* Guayaquil, **1:** 1–33.
11. Tang, F., Chang, H., Huang, Y., and Wang, K., 1957. Studies on the etiology of trachoma with special reference to isolation of the virus in chick embryo. *Chinese M. J.*, **75:** 429–447.
12. Cox, H. R., 1941. Cultivation of rickettsiae of the Rocky Mountain spotted fever, typhus, and Q fever groups in the embryonic tissues of developing chicks. *Science*, **94:** 399–403.
13. Collier, L. H., and Sowa, J., 1958. Isolation of trachoma virus in embryonate eggs. *Lancet*, **1:** 993–996.
14. Collier, L. H., Duke-Elder, S., and Jones, B. R., 1958. Experimental trachoma produced by cultured virus. *Brit. J. Ophth.*, **42:** 705–720.
15. Giroud, P., Renoux, G., and Nataf, R., 1958. I. Cultures de produits trachomateux sur souris, merions, gerbilles et oeufs embryonnes. *Arch. l'Inst. Pasteur* Tunis, **35:** 327–332.
16. World Health Organization. Expert Committee on Trachoma. First report, Tech. Rep. Ser. 1952, **59:** 4.
17. World Health Organization. Expert Committee on Trachoma. Second report, Tech. Rep. Ser., 1956, **106:**
18. Thygeson, Phillips, 1958. Trachoma Manual and Atlas, *Pub. Health Service Publication* No. 541, Govt. Printing Office, Wash.
19. Bovarnick, M. R., Miller, J. C., and Snyder, J. C., 1950. The influence of certain salts, amino acids, sugars and proteins on the stability of rickettsiae. *J. Bact.*, **59:** 509–522.
20. Scherer, W. F., 1953. The utilization of a pure strain of mammalian cells (Earle) for the cultivation of viruses in vitro. I. Multiplication of pseudorabies and herpes simplex viruses. *Am. J. Path.*, **29:** 113–137.
21. Macchiavello, A., 1937. Estudios sobre tifus exantematico. III. Un nuevo método para teñir rickettsia. *Rev. chilena hig. y med. prev.*, **1:** 101–106.
22. Bell, S. D., Jr., Snyder, J. C., and Murray, E. S., 1959. Immunization of mice against toxic doses of homologous elementary bodies of trachoma. *Science*, **130:** 626–627.
23. Terzin, A. L., 1958. Psittacosis-ornithosis-mammalian pneumonitis (POMP) viruses in man, mammals and birds. *J. Hyg., Epi., Micro. & Immunol.*, **2:** 129–142.
24. Bedson, S. P., Barwell, C. F., King, E. J., and Bishop, L. W. J., 1949. The laboratory diagnosis of lymphogranuloma venereum. *J. Clin. Path.*, **2:** 241–249.
25. Dienes, L., and Weinberger, H. J., 1951. The L forms of bacteria. *Bact. Rev.*, **15:** 245–288.
26. Chang, R. S., 1954. Continuous subcultivation of epithelial-like cells from normal human tissues. *Proc. Soc. Exp. Biol. & Med.*, **87:** 440–443.
27. Moore, A. E., Sabachewsky, L., and Toolan, H. W., 1955. Culture characteristics of four permanent lines of human cancer cells. *Cancer Res.*, **15:** 598–602.
28. Murray, E. S., Bell, S. D., Jr., and Allen, H. A. Trachoma in a laboratory worker. To be published.
29. Bell, S. D., Jr., and Murray, E. S. Unpublished observations.

THE NATURE OF THE GASTROINTESTINAL LESION IN ASIATIC CHOLERA AND ITS RELATION TO PATHOGENESIS: A BIOPSY STUDY

E. J. GANGAROSA,* W. R. BEISEL,* CHANYO BENYAJATI,† H. SPRINZ,* AND PRAPONT PIYARATN‡

The classical concept of the intestinal lesion in Asiatic cholera was formulated in 1879 by Virchow[1] who demonstrated villi denuded of epithelium in the small bowel in postmortem studies. This finding was confirmed by Koch in 1887[2] and by Fraenkel[3] in autopsied victims of the 1892 epidemic in Hamburg, Germany. Deycke[4] in the same year presented a convincing argument that epithelium desquamation was an intravital phenomenon by demonstrating these same changes in patients autopsied within an hour of death. In 1916 Stoerk[5] suggested that this process took place by a process of detachment or dissection of sheets or strips of epithelial cells from the basement membrane.

Burnet and Stone[6] supported this concept by demonstrating in vitro an actively desquamating effect on intestinal mucosa of guinea pigs and rabbits by a mucolytic agent from filtrates of cholera cultures. They suggested that such an agent might be responsible for the desquamation of the intestinal epithelium in Asiatic cholera. Thus the current textbook concept of the pathogenesis of cholera was formulated to explain desquamation of the epithelium by a mucolytic toxin resulting in the loss of a prodigious amount of fluid across denuded villi as in a severe burn.[7]

This explanation has not been completely accepted. Cohnheim,[8] Osler,[9] and more recently Goodpasture[10] have maintained that epithelial desquamation in cholera is not an intravital phenomenon but rather is due to postmortem autolysis, and Dutta[11] presented evidence in a large series that desquamation occurs rarely in cholera even after death.

The availability of the peroral biopsy instrument of Crosby[12] made it possible to investigate the pathology of the gastrointestinal tract in a serial manner during the course of acute Asiatic cholera, without artifacts induced by postmortem changes. This study was done during the 1959 epidemic in Bangkok, Thailand and in the endemic area of Calcutta, India.

* Walter Reed Army Institute of Research, Washington, D. C.
† Department of Medicine, Chulalongkorn Hospital Medical School, Bangkok, Thailand.
‡ Department of Pathology, Chulalongkorn Hospital Medical School, Bangkok, Thailand.

MATERIALS AND METHODS

Nine patients with Asiatic cholera were included in this study: eight were of Thai and Chinese nationality from the medical wards of the Chulalongkorn Hospital Medical School in Bangkok, Thailand and the ninth was an Indian from the Infectious Disease Hospital in Calcutta, India. The clinical diagnosis was confirmed bacteriologically in each case (Ogawa serotype), and in addition fluid obtained from the biopsy site was positive by cultural tests for Vibrio cholerae (*V. comma*) in three of these patients. Table 1 summarizes the clinical and laboratory data of these patients on admission, all of whom showed most of the features of classical cholera. An attempt was made to study serial gastrointestinal changes; for example, in one patient a total of five biopsies was obtained, the first on the 2nd day of illness and the last 3 weeks after full recovery. Nineteen biopsies were obtained from these nine patients, with specimens from the stomach, duodenum, jejunum, upper ileum, and colon. Two patients were biopsied on the 1st day of clinical illness, five on the 2nd day, two on the 3rd day, and one on the 4th day. Convalescent specimens were obtained from five patients, three on the 6th day, one on the 8th day and one on the 27th day.

As controls, asymptomatic adults who were incidentally found to be carriers of *V. comma*, and patients with acute diarrhea from whose stools no pathogenic organism was cultured, were also studied. Thirty-eight biopsy attempts were made, 29 of which were successful. There were no ill effects in any of the patients from the biopsy procedure. Table 2 indicates the source and site of the biopsy specimens from the study groups.

The biopsy instrument and technic have been described by Crosby[12] and Smith *et al.*[13] For colon biopsies a new technic was used in which the polyethylene tubing of a Crosby capsule was threaded through the proximal opening of a 3-

foot, flexible, rubber, rectal tube. The body of the capsule was positioned in the long axis of the rectal tube and firmly secured with strips of adhesive tape. After lubricating with petrolatum the capsule and tubing were advanced cautiously through the rectum to a distance of 24 to 30 inches. The details of this method will be reported elsewhere. After the biopsy instrument was triggered it was withdrawn as rapidly as possible, permitting specimens from any site to be fixed in filtered, neutral, buffered formalin (2% sodium acetate, 10% formalin) within 1 to 3 minutes. The specimens were embedded in paraffin and stained with hematoxylin and eosin (H & E). Additional sections were prepared with periodic acid-Schiff reaction (PAS) for basement membrane detail and mucin, alcian blue stain for acid mucopolysaccharides, and the Bodian stain for argentophil granules.

In an attempt to evaluate the character and amount of cellular material in the "rice water" appearing in cholera stools 10-ml specimens were centrifuged at 3000 rpm for 10 minutes. The clear supernatant fluid was discarded and the centrifugate was resuspended in 1 to 2 ml of human plasma. The plasma was clotted by the addition of calcium. The clot containing the cholera stool centrifugate which had produced the "rice water" appearance was then embedded in paraffin; sections were prepared, stained with H & E and studied for histological detail.

RESULTS

The pathological findings in the gastrointestinal tract in Asiatic cholera were those of an acute diffuse enteritis involving principally the small bowel (Fig. 1a, compare with normal Fig. 1b) but also the stomach and colon. The epithelium was found to be intact throughout, albeit altered. At the height of the disease there was a well advanced enteritis which gradually subsided over a period of weeks. The enteritis was characterized by three interdependent processes (inflammatory, reparative and degenerative) with the following features.

There was an inflammatory response of the mesenchymal tissues of the lamina propria of the intestinal mucosa with engorgement of the capillaries and dilatation of the central lacteal. This is the microscopic counterpart to the well known gross findings of vascular engorgement of the small intestine, particularly the ileum. The inflammatory cellular infiltration was moderately severe and was composed of predominantly mononuclear cells, plasma cells, lymphocytes and monocytes with a small number of eosinophils. There was also a migration of these mononuclear cells into the epithelium (Fig. 2). Neutrophilic leukocytes were conspicuous by their absence.

The second element in the histological picture was reparative with plentiful mitoses. This was combined with a severe degree of goblet-cell transformation of the young chief cells located within the crypts of Lieberkuhn and a progressive exhaustion atrophy of the older cells lining the villi (Figs. 3 and 4). This was interpreted as a compensatory hyperplasia of the intestinal epithelial cells in response to a precipitous maturation of the epithelium.

TABLE 1

Clinical features on admission in cholera study group

Case	Age	Sex	History			Admission physical exam				Lab. data	
			Duration of diarrhea(hr)	Muscle cramps	Urine suppression	B.P.	Severe dehydration	"Washer-hands" signs	Rice water stool	W.B.S.G.*	Hct.
1	23	F	14	+	+	0/0	+	+	+	1.068	55
2	39	M	24	+	+	0/0	+	+	+	1.072	65
3	7	M	5	0		0/0	+	0	+		
4	48	M	10	+	0	0/0	+	+	+	1.070	65
5	62	M	16	+	+	0/0	+	+	+	1.071	62
6	52	F	18	+	0	90/70	+	+	+	1.065	60
7	27	M	18	0	+	96/10	+	+	+	1.068	54
8	48	M	14	+	0	120/100	+	+	+		
9	45	M	6	+	+	0/0	+		+		

* Whole blood specific gravity.

TABLE 2

Biopsy specimens—source and site

Study group	Number of		Site of biopsies		
	Patients	Biopsies	Stomach	Small bowel	Colon
1. Asiatic cholera	9	19	2	14	4
2. Nonspecific diarrhea	8	8	0	5	3
3. Asymptomatic carriers of *V. comma*	2	2	1	1	0
Totals	19	29	3	20	7

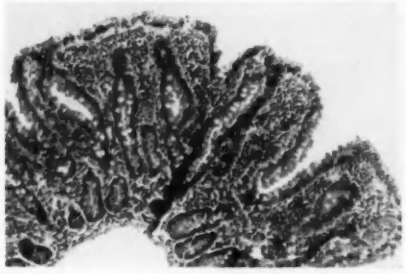


FIG. 1a. Case number 1, H. & E. × 100, 4th day of illness. Small intestinal biopsy (whole thickness of mucosa, probably upper ileum). Note the intact epithelial lining in intimate contact with the lamina propria, the increased cellularity of the latter, the increase in goblet cells in the more basal portion of the villi, the fraying of the free margin and unevenness of staining of the epithelium. Note also the fusion and blunting of several of the villi.

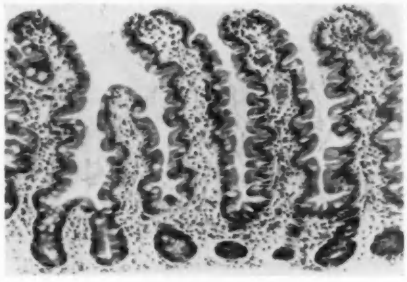


FIG. 1b. Normal North American subject. H. & E. × 100, probably upper ileum. Compare the shape, height and cellularity of individual villi with Figure 1a, note sharpness of free margin and normal number and distribution of goblet cells, and even staining of the cytoplasm of the epithelium.

FIG. 2. Case number 1, 4th day of illness, same biopsy specimen as Fig. 1a. H. & E., × 462, upper ileum. Compare with Figure 1b and the convalescent specimen of the same patient, Figure 8a and b, and note the mononuclear-cell inflammatory infiltrate and migration of these cells into the epithelium, the degenerative changes in the epithelial lining manifested by irregular staining of the cytoplasm, bunching and spindling of nuclei, presence of vacuoles, and loss of definition of the brush border.

The third element featured degenerative changes particularly noticeable in the epithelium lining the tips of the villi. Here the cells had become very flattened and frayed. The nuclei of many of these cells had lost their definition, had decreased staining qualities, and the cytoplasm contained numerous vacuoles. We feel that these changes are associated with accelerated shedding of epithelial cells reflecting a rapid cellular turnover. Rapid turnover could also be inferred from the increased numbers of mitoses in the crypts. Accelerated shedding of these cells accounts for the "ghost" cells in the rice water stool centrifugate to be described subsequently. There were also degenerative changes in the basement membrane with formation of microscopic vacuoles and spaces (Fig. 5). These may be the precursors of the subepithelial edema noted in postmortem specimens by Goodpasture,[10] and the detachment of the epithelium from the basement membrane noted at autopsy by Stoerk.[5] The lamina propria itself exhibited karyorrhexis and lysis of mononuclear cells and the occurrence of an amorphous

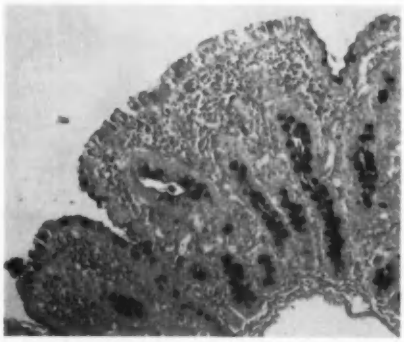


FIG. 3. Case number 1, same biopsy specimen as Figures 1a and 2. PAS, × 115, probably upper ileum. Compare this acute specimen with the convalescent specimen, Figure 4, and note distribution of PAS-positive mucus (appearing black). Note marked increase of goblet cells within crypts and at the base of villi and almost complete absence at the luminal surface of the villi. The fusion of villi is evident.

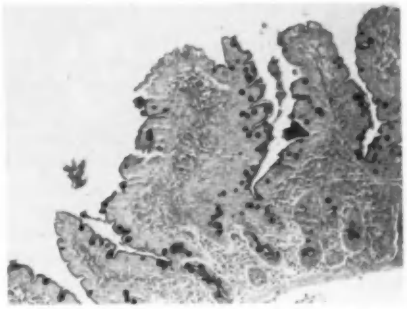


FIG. 4. Case number 1, same biospy specimen as Figure 8a and b, obtained in convalescence, 27 days after onset of cholera. PAS, × 115, probably upper ileum. Note near-normal distribution of goblet cells and delicate PAS-positive mucus covering of the free surface.

precipitate (Fig. 6). This precipitate is a nonspecific abnormal finding and has been noted by Sprinz,[14] in the exudative enteropathy of Gordon,[15] in malabsorption states, and in experimental cholera in guinea pigs.

An important additional finding in our noncholera specimens, as well as in the biopsies obtained from cholera patients, was evidence of a chronic atrophic enteritis with fusion and blunting of intestinal villi indicative of a sprue-like syndrome (Figs. 1, 3 and 4). These chronic changes, though constant in a given patient even in convalescence, were variable in intensity within the group. In our cholera patients the

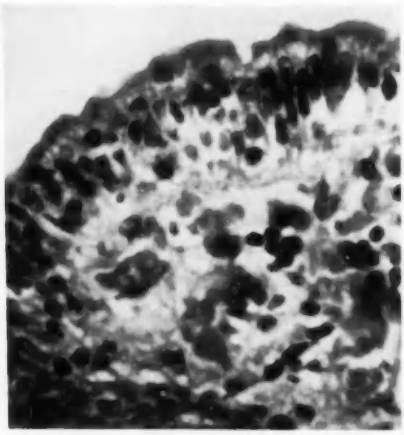


FIG. 5. Case number 5 (see Table 1) first day of illness. H. & E., × 770, probably upper ileum. Note intact epithelial surface intimately covering the lamina propria, the variability of staining of the epithelium, the numerous vacuoles in the cytoplasm, particularly in the subnuclear position, the ill-defined and seemingly edematous basement membrane. There is also migration of mononuclear cells into the epithelium.

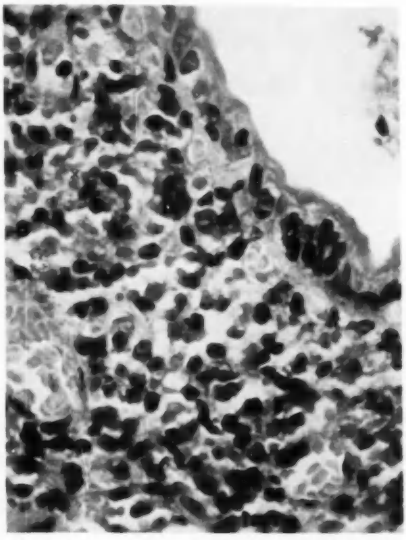


FIG. 6. Case number 1, 4th day of illness, same biopsy specimen as Figures 1a and 2. H. & E., × 770. Note intact epithelial lining with marked variability of epithelial cells showing degenerative changes. Note hypercellularity of lamina propria, karyorrhexis and presence of proteinaceous, amorphous precipitate, the latter indicated by the letter X.

acute enteritis was superimposed on the chronic atrophic process.

All of these features are illustrated by the findings in the serial biopsies reported in the following two cases. The remaining seven patients showed the same pathological features. The principal clinical admission findings in these cases are summarized in Table 1.

Cholera Patients

Case 1. A 23-year-old Thai woman was hospitalized 14 hours after the onset of severe Asiatic cholera manifested by a profuse watery diarrhea and profound shock (see Table 1). On the 2nd day of illness a biopsy was obtained from the gastric mucosa which contained pyloric-type glands and portions of the muscularis mucosa. The epithelium was intact and there was microscopic evidence of an acute and chronic gastritis. Chronic inflammatory cells, mainly lymphocytes, mononuclear cells, and plasma cells were present throughout the lamina propria, with transmigration of these cells across the epithelium. Leukocytes were rare. The lamina propria appeared edematous and contained an amorphous proteinaceous precipitate. Degenerative changes were noted in the mucosal cells, evidenced by alterations in the staining property, and vacuolization and loss of definition of nuclei. The pyloric glands were distended with mucus. In the depths of these glands a distinct increase in the number of mucus-producing cells was evident with a moderate degree of reactive pleomorphism of the nuclei. But, closer to the lumen, a progressive exhaustion of mucus production of these cells was apparent (Figs. 7a and 7b).

The following day the patient lost 5 liters of fluid through continuing diarrhea, and the stool bacteriology was still positive for *V. comma*. A second biopsy from the same portion of the stomach again showed an intact muscosa with evidence of acute and chronic gastritis. The same microscopic abnormalities noted in the previous specimen were present.

By the 4th day of illness, the stool culture was still positive but the diarrhea had virtually ceased. A biopsy from the jejunum or upper ileum included the entire thickness of the mucosa and revealed an active enteritis. The surface epithelium was intact and fully attached to the lamina propria. The latter was infiltrated with plasma cells, lymphocytes, and histiocytes.

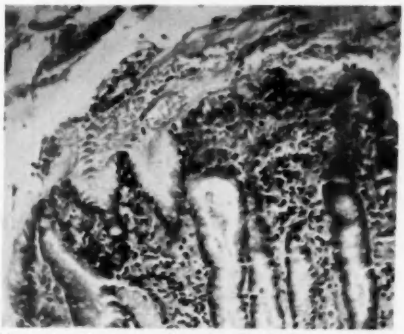


FIG. 7a. Case number 1, H. & E., × 231, 2nd day of illness, stomach, pyloric region. Note acute gastritis with cellular infiltrate and edema of the lamina propria, marked degenerative change of the surface epithelium with loss of definition of cell borders of individual cells and excess production of apparently altered mucus on the luminal surface.

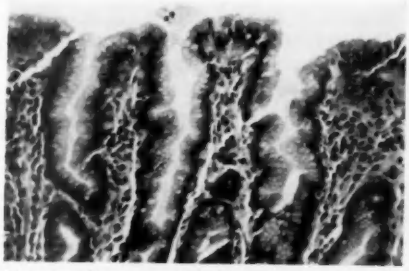


FIG. 7b. Compare with Figure 7a, same type of preparation and magnification of identical area, essentially normal North American subject.

Some of these cells had transmigrated across the mucosa. A moderate number of eosinophilic leukocytes were present but segmented leukocytes were distinctly rare. Some of the cells in the lamina propria stained only in a faint outline and, in addition, a precipitate similar to that noted in the lamina propria of the stomach was present. The epithelium was characterized by considerable pleomorphism manifested by variable intensity of staining and spacing of nuclei. Small vacuoles were noted in some of the nuclei while others showed a condensation of the chromatin material at the nuclear membrane. Some of these nuclei were spindle-shaped and bunched together while others were compressed against the base of the cells by large inclusions. While in the H & E preparation these inclusions looked alike and resembled goblets, *e.g.*, aggregates of mucinogen particles, special stains with PAS,

and even more so with alcian blue, revealed the presence of carbohydrate and acid mucopolysaccharides in only some of the inclusions, particularly in those located in the crypts and close to the base of the villi. These alterations suggest that although goblet cells are greatly increased, apparently in response to the inflammatory stimulus, they are producing an imperfectly constituted mucus, as evidenced by the relative absence of acid mucopolysaccharides in the majority of goblet cells. We interpret this as possibly reflecting more rapid mucus formation leading to exhaustion of mucus-producing cells. In addition the biopsy showed the marked fusion and blunting of villi as illustrated in Figure 1. Considering the approximate site of the biopsy the thickness of the mucosa was somewhat decreased. The changes in the villi and the atrophy of the mucosa suggested a sprue-like condition.

The fourth specimen from this patient was obtained from the upper ileum on the 6th day after the onset of her disease when stool bacteriology was negative and the patient was clinically well. The biopsy included mucosa and muscularis mucosa. There was evidence of an enteritis in the healing phase. The mucosal lining was completely intact and there was transmigration of inflammatory cells but to a lesser degree then previously noted, with less pleomorphism of the mucosal cells. The brush border was discernible in many places and appeared normal. There was still an increase in mucus-producing cells and considerable bunching of the epithelial nuclei. The nuclei were in general ovoid in shape and some were moved toward the midportion of the cells. The chromatin was delicately reticulated and some vacuoles were noted within the nuclei. Most of the lining cells contained vacuoles and granules in their cytoplasm. True goblet cells were absent from the tips of the villi but were prominent at the bases of the villi and in the crypts of Lieberkuhn. Paneth's cells were inconspicuous just as they were in the preceding biopsy. The lamina propria was infiltrated with a moderate number of predominantly mononuclear cells and plasma cells. There were also a moderate number of eosinophils. The proteinaceous precipitate previously noted was also present but to a lesser extent. While the mucosal surface layer was intact the basement membrane in several places was indistinct. In those areas where capillaries abutted the epithelium, it was noted that the space between the capillaries and the nucleus appeared widened and there were some subnuclear vacuoles. The sprue-like changes persisted.

The fifth and last biopsy from this patient was obtained from the jejunum 27 days after the onset of cholera. The patient was clinically well and had returned to work as a laborer in a lumber mill. Stool culture was negative for *Vibrio comma*. The specimen included the entire thickness of the mucosa and showed the same chronic changes, which resembled a malabsorptive state and resolution of the acute enteritis. The villi were variable in their appearance; some appeared normal while others were blunted and fused (Fig. 4). The mucosal lining was intact and the brush border well preserved (Fig. 8). In places, however, there was moderate pleomorphism of the mucosal lining cells and unevenness of the cytoplasm with a number of the nuclei moved from the basal into the midposition within the cell. The lamina propria showed a slight increase in cellularity including a moderate number of eosinophils. The precipitate in the lamina propria, described above, was again noted but was greatly diminished.

Case 2. A biopsy from the upper ileum was obtained on the first day of illness in a 39-year-old Thai male laborer who presented the classic features of severe Asiatic cholera (see Table 1). Microscopic changes of an acute enteritis were superimposed on a chronic process suggestive of a malabsorptive state. The latter process was manifested by blunting and fusion of villi and atrophy of the mucosa. The epithelium was intact throughout, and the brush border appeared normal. Occasionally, small vacuoles were present in the region of the basement membrane and at the base of the chief cells. Paneth cells were seen in the crypts of Lieberkuhn and mitotic figures were plentiful.

The acute enteritis was manifested by a moderately severe infiltration of the lamina propria predominantly by lymphocytes and plasma cells, with transmigration of these cells through the epithelial lining membrane. In addition, the lamina propria contained occasional nuclear debris, a proteinaceous precipitate, and an occasional small hemorrhage.

A colon biopsy, also obtained on the 1st day of illness, revealed a mild subacute colitis manifested by focal hemorrhages into the lamina

FIG. 8a

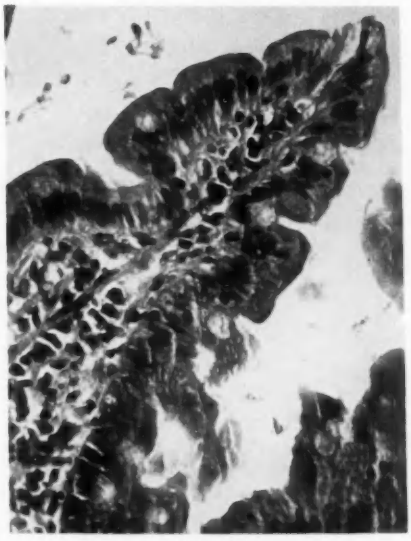


FIG. 8b

FIG. 8a and b. Case number 1, H. & E., × 232 and × 462, respectively, 27th day after onset, probably upper ileum, whole thickness of mucosa. Compare with Figure 2, acute specimen, and note sharpness of brush border, uniformity of cytoplasm and epithelial nuclei. An increased cellular infiltrate has persisted in the lamina propria and may be associated with a presumably nutritional chronic atrophic enteritis.

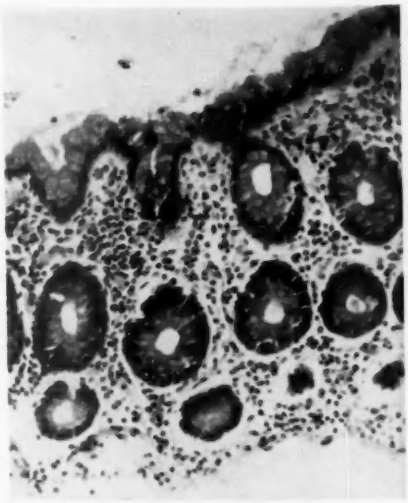


FIG. 9. Case number 2, H. & E., ×231, 1st day of illness, colon. Note intact and essentially regular mucosa and slight inflammatory cell infiltrate in the lamina propria.

propria and accumulation of plasma cells and a few leukocytes. The mucosa was intact (Fig. 9).

A biopsy was obtained from the jejunum 6 days after the onset of disease when the patient was clinically well with negative stool cultures for *Vibrio comma* for the preceding 3 days. Although the same chronic process of fused and blunted villi persisted, the acute inflammatory response had greatly diminished in intensity. The mucosal lining was completely intact and the brush border was well preserved. Goblet cells were slightly more prominent than usual. The epithelial cells were rather narrow and elongated and appeared to be crowded. Vacuole formation was still noted between the basement membrane and the epithelial cells, and occasional nuclear debris was seen in this location. The lamina propria of the intestinal villi showed an increase in ground substance suggesting mild edema. The same amorphous precipitate noted in other specimens was also present. A slight increase in predominantly mononuclear cells, plasma cells and lymphocytes was apparent. Segmented leukocytes were not seen. Within the crypts the Paneth cells were normal but there was a slight increase in goblet cells and in mitotic figures. In summary, the convalescent biopsy showed changes essentially similar to those seen in the acute specimen except

that the inflammatory response was much less severe.

The nonspecific nature of the histological changes seen in patients with clinical cholera did not permit a pathological differentiation when compared with the nonspecific diarrhea cases in this study. This is in contrast to the appearance of certain bowel lesions such as typhoidal or tuberculous granulomata which permit a specific pathological diagnosis.

Nonspecific changes similar in character but not in degree were noted in the biopsies of patients suffering from diarrhea of undetermined etiology. This emphasizes the point that the types of histological reactions which can occur in the small intestine are limited, at least as far as one can determine with present tools and technics. Histologically, the main difference between nonspecific enteritis and the enteritis of cholera was in the degree of tissue response, particularly in the intestinal epithelium which was more severely involved in cholera. Excess mucus production was noted in the crypts of Lieberkuhn in both conditions. The appearance of an exhaustion atrophy was seen in the more distal epithelial cells and was followed by flattening and bunching together of the cells at the tips of the villi. The degenerative changes and the reactive pleomorphism in the epithelial cells were, for all purposes, identical cytologically. The changes in the basement membrane and in the lamina propria were also similar. The possibility exists that some of these undiagnosed cases of diarrhea in this epidemic of cholera may have been unrecognized mild cases of cholera.

Asymptomatic Carriers

A gastric biopsy from an asymptomatic carrier of *Vibrio comma*, a Thai Army recruit, revealed a focal, moderate, subacute gastritis involving pyloric-type glands. This was manifested by an unevenly distributed infiltration in the lamina propria of an increased number of mononuclear cells. In places the inflammatory infiltrate destroyed or partially obscured the cells of the mucous neck glands. There was an occasional regular lymph follicle in the mucosa. The gastric pits were regular as were the pyloric glands. These abnormal findings are considered nonspecific

A biopsy from the duodenum of another Thai Army enlisted man, an asymptomatic carrier of *Vibrio comma*, showed a normal epithelium and Brunner's glands. The villi were shorter than normal and there was some fusion. The number of cells in the lamina propria was slightly increased. The findings reflected chronic atrophic duodenitis as may be seen in a malabsorptive state.

Description of Cholera Stool Precipitate

The principal component of the solid material which gave the cholera stools a rice-water appearance was found to be mucus. Occasionally, a nest of mononuclear cells and lymphocytes could be seen. Infrequently epithelial "ghost" cells, individually and sometimes in groups of six to eight connected cells, were encountered.

DISCUSSION

The classical concept of the pathogenesis of Asiatic cholera is that the intestinal epithelium is lost during life resulting in the prodigious loss of fluid across denuded villi. A mucolytic enzyme derived from the vibrio has been thought by many to be responsible for the epithelial desquamation. The present study, on the other hand, demonstrates that the intestinal epithelium is intact throughout the course of the disease.

The discrepancy between our findings and those from which the classical concept was derived is explained by the fact that our study is based on biopsy specimens in contrast to the often autolyzed postmortem material which was available to others. Deycke[4] and Goodpasture[10] based their findings on material which was fixed within an hour of death, yet they reached different conclusions; the former investigator demonstrated desquamation and the latter that the epithelium was intact during life. To obviate the artifacts resulting after death our biopsy specimens were placed in fixative within 1 to 3 minutes after they were obtained. Previous observations have established that during the short time interval between triggering of the biopsy device and placing the specimen into fixative the autolytic changes are minimal, and that the smallest submicroscopic structures and organelles can be demonstrated with the electron microscope.[14, 16]

In recent years considerable progress has been made in the understanding of the normal intestine and the pathology of different intestinal diseases because of the introduction of various biopsy devices, the Wood[17] and the Shiner[18] instruments

and finally the Crosby capsule. The availability of intestinal biopsy specimens has introduced a new era in the understanding of intestinal pathophysiology to supplement the knowledge gained in the past. However, there are some limitations inherent in the biopsy technic. Only a small specimen of mucosa can be obtained. The exact site of the biopsy cannot be controlled and the lower part of the ileum is more difficult to reach. These factors were limitations in our study and prevented us from obtaining specimens from the lower ileum where the pathological condition in cholera is believed to be most severe. To circumvent these limitations, we obtained serial specimens from all other parts of the intestinal tract involved in the disease. We believe that this study proves that the mucosa is intact during life and thus confirms the essential postulates of Cohnheim, Osler, and Goodpasture. Our findings are supported by the physiological studies discussed below which further indicate that the intestinal mucosa is intact throughout its entire length, including the lower ileum.

It is our belief that the findings of epithelial denudation by Virchow, Fraenkel, and Deycke; of epithelial detachment by Stoerk; and of subepithelial edema by Goodpasture represent postmortem artifacts due to autolysis from digestive enzymes and proliferating bacteria. These are nonspecific postmortem findings that may be seen following death from many causes. Dutta[11] has shown that only two of his 64 autopsied cholera victims had epithelial denudation in the small bowel. It must, therefore, be inferred that an enzyme such as the "mucolytic enzyme" of Burnet and Stone[6] does not cause desquamation during life in human Asiatic cholera. Although desquamation does not occur, it is still entirely possible that this enzyme may be involved in the pathogenesis of cholera through some other mechanism.

The finding of desquamated epithelial cells in the "rice-water" stools of cholera victims has been cited as evidence of the frequent occurrence of an intravital detachment of the intestinal mucosa.[5] That epithelial cells, sometimes in layers, are found in the stools of cholera victims has been confirmed in this study; however, they are seen infrequently, and their numbers are not in excess of what might be anticipated from the expected rapid turnover of epithelial cells in the normal intestinal mucosa.[19] Shedding of cells from the tips of intestinal villi is a normal process[20] which we believe is accelerated in cholera. These cells are usually broken down within the bowel lumen, but the rapid flow of diarrheal fluid in cholera probably accounts for the appearance of these cells in the stool, and it is our belief that their presence in the stool does not reflect denudation of whole villi occurring during life. In fact, the principal component which accounts for the rice-water appearance of the cholera stool is neither cells nor cellular debris, but mucus. Excessive mucus production by the increased goblet cell population suspended in the large volume of essentially isotonic fluid of the cholera stool explains the rice-water appearance.

The serial biopsies provided an understanding of the evolution of the inflammatory, degenerative and reparative processes in the intestinal mucosa in cholera. Over the course of days the inflammatory and degenerative processes subsided while repair continued until full restitution was obtained in late convalescence. It was not possible to correlate histological features with clinical diarrhea. There was continued evidence of severe enteritis even when the patient's diarrhea had ceased and the patient was clinically well. This phenomenon is analogous to the persistence of the roentgenological and morphological alterations in the lung following the occurrence of the "crisis" in lobar pneumonia.

The acute enteritis of cholera was superimposed on a separate chronic pathological process which must have antedated the cholera infection. The chronic changes were characterized by blunting and fusion of villi and mucosal atrophy strongly suggestive of nutritional deficiency or malabsorptive defect. These features persisted even in the convalescent period after the acute inflammatory process had resolved. These findings would suggest the possibility that dietary or nutritional factors may play an important role in the pathogenesis of the disease. It is a well known epidemiological fact that the lower socioeconomic strata are most frequently involved in epidemic outbreaks of cholera and it is in this group that the diet would be most likely to be deficient.

Numerous physiological studies support the concept that the intestinal epithelium is intact in the cholera victim. The failure of intravenously injected Evans Blue Dye (which rapidly attaches itself to serum albumin[21] or radioiodinated polyvinylpyrrolidone (PVP-I^{131})[22] to appear in the

stool in cholera implies that there is virtually no leakage of albumin into the gut. A denuded intestinal surface acting as a "burned surface" would be expected to leak albumin; such is not the case in cholera. Inulin molecules, which diffuse freely throughout the extracellular space but do not enter cells, failed to appear in the cholera stool,[23] thereby giving further evidence of an intact intestinal mucosa.

The ability of the intestinal tract to absorb rapidly and completely molecules of I^{131} and tritiated water placed in the duodenum during active cholera diarrhea[23] gave evidence that all absorptive functions were not lost. The findings of relatively higher potassium and bicarbonate levels in the intestinal fluid, in comparison to simultaneously obtained serum levels,[24] can be interpreted as evidence of active "work" by mucosal cells in acute cholera. All of these studies indicate that not only is the intestinal epithelium intact but also that it must play a major role in establishing the unique characteristics of the cholera stool.

The questions which arise from these studies outnumber the answers given. Future research in this field would, it is hoped, answer the following questions. How does the *Vibrio* initiate the disease? What is the cause of the explosive diarrhea in cholera and its spontaneous cessation in the presence of continued enteritis? By what mechanism is the fluid actually lost? Is there a yet-unrecognized, specific, morphological lesion in cholera or is the clinical picture related to a primary physiological defect? Can the fluid losses be related to an imbalance between absorptive and secretory phenomena, either active or passive, or to a more basic change in vascular or hydrostatic balances? Is there an acquired predisposition related to diet or nutrition which makes an individual susceptible to cholera? What are the factors which account for the spontaneous elimination of the *Vibrio*? These and many other problems provide a challenge to future clinical investigation.

SUMMARY

1. A serial intestinal biopsy study of patients with classical epidemic and endemic Asiatic cholera demonstrates that the intestinal epithelium in this disease is intact, contrary to the classical textbook concept that a mucolytic enzyme causes desquamation of the epithelium during life.

2. The histological abnormalities of the small bowel in cholera revealed an acute enteritis manifested by a mononuclear cell inflammatory exudate, vascular congestion, marked goblet cell hyperplasia with eventual exhaustion atrophy, and increased "turn-over" of epithelial cells. Except in degree, the lesion in cholera could not be differentiated from that in cases of nonspecific diarrhea observed during this epidemic.

3. The additional finding of a separate, underlying, chronic, atrophic enteritis suggested that a dietary or nutritional factor may be important in predisposing an individual to cholera.

4. Mucus rather than desquamated cells gives the "rice-water" stool its characteristic appearance.

ACKNOWLEDGMENTS

To Prof. Luang Prakit Vejasakti, Dr. Prayat Laksanaphuk, Dr. Ampan Tasniyavej, Dr. Abhaya Chamuni, Dr. Somsakti Dejakaisaya, Prof. Pirom Suvanatemiya, Dr. Sira Sirisambandh, Dr. Luang Binbakya and the residents, interns and nurses of the Chulalongkorn Medical College, Bangkok, Thailand; Prof. K. C. Basu Mallik of the Nilratan Sircar Medical College, Calcutta, India and Dr. R. N. Chaudhuri of the School of Tropical Medicine, Calcutta, India for assistance and encouragement in this work.

To Dr. A. Mondal of the Infectious Disease Hospital, Calcutta, India for permission to study and biopsy his patient.

To Col. Gottlieb Orth, MC, Capt. Eugene Blair, MSC and Col. B. H. Sullivan, Jr., MC, U. S. Army; Col. Chamras Thephusdin, MC, Royal Thai Army; Capt. Robert Phillips and Commander Raymond Watten, MC, U. S. Navy and Lt. Col. Oscar Felsenfeld, Dr. Samuel Formal, Dr. Richard Finkelstein and Dr. Geoffrey Edsall of Walter Reed Army Institute of Research for their comments and suggestions.

To Dr. Rolf Freter and Dr. Harry Smith of the Jefferson University Research Unit for bacteriologic support, and Mr. F. Y. Halsey of Walter Reed Army Institute of Research for assistance in the preparation of the manuscript, and the Medical Audio Visual Department, Walter Reed Army Institute of Research for photomicrography.

REFERENCES

1. Virchow, R., 1879. Gesammelte Abhandlungen auf dem Gebiete der öffentlichen Medizin, Berlin, **1**: 151.

2. Koch, R., 1892. *Arbt. kaiserl. Ges.*, (1887), **3:** 156.
3. Fraenkel, E., 1893. Über Choleraleichenbefunde. *Deutsche med. Wchnschr.*, **19:** 157.
4. Deycke, G., 1892. Über histologische und bacilläre Verhaltnisse im Choleradarm. *Deutsche med. Wchnschr.*, **18:** 1048.
5. Stoerk, O., 1916. Über Cholera. *Beitr. path. Anat.*, **62:** 121.
6. Burnet, F. M., and Stone, J. D., 1947. The mucinase of *Vibrio cholera*. *Australian J. Exper. Biol. & M. Sc.*, **26:** 71–80.
7. Gradwohl, R. B. H., Benitez Soto, L., and Felsenfeld, O., 1951. *Clinical Tropical Medicine*, pp. 477–506. St. Louis. C. V. Mosby Co.
8. Cohnheim, J. F., 1889–90. Lectures on general pathology—*A Handbook for Practitioners and Students*, London (Translated from the 2nd German edition by McKee, A. B.).
9. Osler, W., 1892. *The Principles and Practice of Medicine*, p. 121. New York: D. Appleton and Co.
10. Goodpasture, E. W., 1923. Histopathology of intestine in cholera. *Philippine J. Sci.*, (Sect. B), **22:** 413–423.
11. Dutta, S. K., 1941. Investigation on the behavior of kidney in cholera. *Calcutta M. J.*, **38:** 621–636.
12. Crosby, W. H., and Kugler, H. W., 1957. Intraluminal biopsy of the small intestine. *Am. J. Digest. Dis.*, **2:** 236–241.
13. Smith, R. B. W., Sprinz, H., Crosby, W. H., and Sullivan, B. H., 1958. Peroral small bowel biopsy. *Am. J. Med.*, **25:** 391–394.
14. Sprinz, H. Personal observations.
15. Gordon, R. S., 1959. Exudative enteropathy. *Lancet*, **1:** 325–326.
16. Hartman, R. E., Smith, R. B. W., Hartman, R. S., Butterworth, C. E., and Molesworth, J. M., 1959. The electron microscopy of the human intestinal epithelium obtained with the Crosby intestinal biospy capsule. *J. Biophys. Biochem. Cytol.*, **5:** 171–172.
17. Wood, I. J., Doig, R. K., Motteram, R., Weiden, S., and Moore, A., 1949. The relationship between the secretions of the gastric mucosa and its morphology as shown by biopsy specimens. *Gastroenterology*, **12:** 949–958.
18. Shiner, M., 1956. Jejunal-biopsy tube. *Lancet*, **1:** 85.
19. Leblond, C. P., and Walker, B. E., 1956. Renewal of cell populations. *Physiol. Rev.*, **36:** 255–276.
20. Bizzozero, G., 1888. Über die Regeneration der Elemente der schlauchförmigen Drüsen und des Epithels des Magendarmkanals. *Anat. Anz.*, **3:** 781.
21. Saha, H., and Das, A., 1952. Observations on the nature of cholera stools. *J. Indian M. A.*, **21:** 464–467.
22. Gordon, R. S. Unpublished data.
23. Watten, R. H., Beisel, W. R., Lee, C. Y., Gangarosa, E. J., and Phillips, R. A. Renal and bowel responses to bicarbonate loading during acute Asiatic cholera. In preparation.
24. Watten, R. H., Morgan, F. M., Songkhla, Y., Vanikiati, B., and Phillips, R. A. Water and electrolyte balance studies in cholera. In press.

SURVIVAL, AND PROTECTION AGAINST CHLORINATION, OF HUMAN ENTERIC PATHOGENS IN FREE-LIVING NEMATODES ISOLATED FROM WATER SUPPLIES

SHIH LU CHANG, GERALD BERG, NORMAN A. CLARKE, AND PAUL W. KABLER

Robert A. Taft Sanitary Engineering Center, Bureau of State Services, Public Health Service, U. S. Department of Health, Education, and Welfare, Cincinnati, Ohio

In a previous communication[1] the persistent occurrence of the free-living nematode, *Diplogaster nudicapitatus*, in a city water supply was reported. In a current survey on the occurrence of free-living nematodes in city water supplies in the United States (which will be fully reported upon completion), it has been revealed that of the 22 supplies examined so far, 16 showed live, free-living nematodes falling into the following genera: *Rhabditis*, *Cheilobus*, *Diplogaster*, *Monhystera*, *Aphelenchus*, *Cephalobus*, *Dorylaimus* and *Turbatrix*.

While these nematodes are harmless per se when ingested, those of the Rhabiditidae family may possibly serve as carriers of human enteric pathogens. This is based on the facts that members of this family are frequently present in large numbers in trickling filters of municipal sewage treatment plants (unpublished observations of S.L.C.) and that they feed readily on *Proteus mirabilis*, *Aerobacter aerogenes*, *Ae. clocae* and *Pseudomonas* sp. in laboratory cultures.[2]

Early preliminary observations made with these nematodes grown in bacterial cultures indicated that *Salmonella* and *Shigella* organisms survived 3 to 4 days in the worm gut. But the contamination by the original bacteria complicated the determination of the survival of the pathogenic bacteria and the results must be considered as inconclusive.

Since axenic cultures of several species of Rhabiditidae were established in January 1959, attempts were again made to find out how effectively these nematodes feed on human enteric pathogens, how long these can survive in the worm gut, whether any viable pathogens are excreted by the worms, and how much protection against chlorination the carrier-worms can offer to the ingested pathogens.

MATERIALS AND METHODS

The nematodes *D. nudicapitatus* Steiner, 1914 and *C. quadrilabiatus* Cobb, 1924 were employed in the investigation. The former was isolated in June, 1956 from a city water supply[1] and the latter from a similar source in December, 1958. They were grown axenically in a serum-casein-yeast-extract acid-phosphate fluid medium described in another report.[2] The average yield at the growth peak in 30 ml T flasks was about 40,000 adults and larvae per culture of the *C. quadrilabiatus* or about 5,000 per culture of the *D. nudicapitatus*.

Suspensions used in the feeding experiments were prepared in buffered dilution water[3] with cultures at the growth peak to contain about 8,000/ml of adults and larvae of the *C. quadrilabiatus* or about 1,000 of the other nematode. The enteric bacteria used in the feeding were *S. typhosa*, *S. paratyphi*, *S. typhimurium*, *Sh. sonnei*, and *Sh. dysenteriae* II, all of which were obtained from Dr. W. H. Ewing of the Communicable Disease Center, Public Health Service. To feed on bacteria, 1 ml of the suspension was mixed with an equal amount of a 0.1% semisolid agar (to increase survival of the worms) and poured on a plate culture of the bacterium in question on a buffered sucrose-dextrose-tryptose agar. This medium has been used for growing the flagellate-ameba *Naegleria gruberi* on *Salmonella* and *Shigella* bacteria[4] and also for growing the present nematodes in bacterial association.[2]

To feed on the enteric viruses, 1 ml of the worm suspension was placed in a 30 ml T flask which contained 5–6 ml of a crude virus suspension having a titer of about 10^8 cytopathic units per ml. The viruses used were the CME-456 strain of Coxsackie A9 and the HEV_1, Wallace strain of Echo 7 (both supplied by Dr. M. Ramos-Alvarez of the Children's Hospital Research Foundation, Cincinnati). Both viruses were grown in monkey kidney tissue cultures and the medium harvested after complete lysis of the epithelial cells was used as the crude virus suspension.

After 2 days of feeding at room temperature (25°–27° C), the worms were washed off the bacterial plates with a small amount of buffered water. The worm-bacterium or the worm-virus suspension, diluted with enough buffered water to give a total volume of about 100 ml, was flocculated with 50–60 ppm of aluminum sulfate to remove dead worms and extraneous matter, including free bacteria and virus. After the floc had settled, the supernate was decanted and centrifuged. The sediment, containing practically all live worms, was resuspended in 10 ml of the buffered water.

To ascertain the survival of the ingested pathogens, the worm suspension prepared above was chlorinated with 10 ppm of free chlorine at pH 6.6–7.2 for 15 minutes. As will be seen later, this treatment destroys any free bacteria or virus remaining in the suspension but has no harmful effect on the worms. After the addition of an excess of sodium thiosulfate to stop the activity of the chlorine, the suspension was finally diluted with enough autoclaved Ohio River water to give about 50 *C. quadrilabiatus* worms or about 10 *D. nudicapitatus* worms per ml. The worm-laden water was stored at room temperature for studying the survival of pathogens in worms. A small amount was used for studying the excretion of viable pathogens by worms.

The survival of pathogens inside the worms was ascertained as follows: Within 2 hours after preparation, a 50-ml sample of the worm-laden water was centrifuged and the worm-sediment ground in a tissue grinder. Suitable dilutions of the ground material were made and plated for viable bacteria or inoculated into monkey kidney tissue culture tubes for virus. Bismuth sulfite agar was used for *S. typhosa* and SS agar for other *Salmonella* and the *Shigella* spp. The cytopathic units of virus were estimated by the most probable number method of Chang *et al.*[8] When necessary, doubtful bacterial colonies were confirmed by fermentation and infrared spectrophotometric characteristics, and nonspecific cytopathic changes in tissue cultures were confirmed by passage. After 24 and 48 hours of standing, the survival of the ingested pathogens was again ascertained.

To determine whether viable bacteria were excreted by the worms, 1 ml of the worm-laden water was spread on each of four plates of the buffered sucrose-dextrose-tryptose agar. The plates were placed in a 35° C moisture-saturated incubator and were examined daily for 5 days for development of bacterial colonies.

To detect viable virus excreted by the worms, 0.5 ml of the worm-laden water was pipetted into each of 8 tubes of the monkey kidney tissue cultures. The inoculated tubes were incubated at 35°C on a roller drum. The constant movement of the medium apparently was effective in preventing the small number of worms from exerting harmful effect on the epithelial cells. Since 70–80% of the worms died within 48 hours under these cultural conditions, the medium was changed 2 days after the worms were introduced and the tubes were examined for 1 week after the change of the medium.

For use in the study of the protection by carrier nematodes of the ingested pathogens against chlorination, the pathogen-fed worm suspension was prepared in the same manner as that used in the survival study except that the free pathogen was not destroyed by chlorine. In fact, some free bacterial or viral suspension was added to the worm suspension so as to show the relative survival of the free and the worm-borne organism. Suspensions thus prepared usually had a *C. quadrilabiatus* worm density of about 2,000 per ml or a *D. nudicapitatus* worm density of about 500 per ml, and a free pathogen density of 5,000–20,000 colonies or cytopathic units per ml.

To chlorine solutions containing free chlorine ranging from 3 to 100 ppm and buffered at pH 6.6-7.2 the worm-pathogen suspension was added in such amounts that the suspension was diluted 100 times. After scheduled intervals of standing at 25° C, 25-ml samples were removed from the respective chlorinated waters for titration of residual chlorine and 50-ml samples were placed in centrifuge tubes containing an excess of sodium thiosulfate. The dechlorinated 50-ml samples were centrifuged and the supernates siphoned off, except that from those with short contact times a 3-ml sample of each supernate was saved for determining the survival of the free pathogen. The worm-laden sediment from each sample was ground in a tissue grinder, except for one drop to be examined for motile worms.

The bismuth sulfite and the SS agar were again used for determining the survival of

Salmonella and *Shigella* spp. The plaque count method of Dulbecco[6] modified by Hsiung and Melnick[7] was used to ascertain the survival of Coxsackie A9 and Echo 7 virus. Doubtful bacterial colonies or nonspecific cytopathic changes, when encountered, were confirmed as before.

In each experiment, a control was prepared with the same worm-pathogen suspension in water without chlorine, and was tested for pathogen contents in the same manner as the test samples. Bacterial or viral densities in both the supernate and the worms of the control were used as indices of concentrations of the pathogen before chlorination.

Sterile precautions were observed throughout the investigations.

RESULTS

Survival of enteric pathogens in nematodes. Results obtained in observations made with the *D. nudicapitatus* and *C. quadrilabiatus* fed on *S. typhosa*, *S. paratyphi*, *Sh. sonnei*, and Coxsackie A9 virus are shown in Table 1 where several points of interest may be noted. First, the survival of these enteric pathogens was not significantly different in these two nematodes. Second, the *S. typhosa* and *Sh. sonnei* showed a comparable survival—5 to 6% survival after 24 hours and less than 0.1% after 48 hours. Third, the *S. paratyphi* and the Coxsackie A9 virus showed a comparable survival which was definitely longer than that of the *S. typhosa* and *Sh. sonnei*—12 to 16% after 24 hours and about 1% after 48 hours. The smaller numbers of organisms ingested by the *C. quadrilabiatus* than by the *D. nudicapitatus* may be explained by the smaller size of the former nematode.

TABLE 1

Survival of S. typhosa, S. paratyphi, Sh. sonnei, *and Coxsackie A9 virus in free-living nematodes suspended in water at 25° C*

Enteric pathogen	Time after feeding in hrs	Survival of bacterium or virus in 100 worms			
		D. nudicapitatus		*C. quadrilabiatus*	
		No.	%	No.	%
S. typhosa	2	8,000	100	1,800	100
	24	480	6	95	5.3
	48	6	0.08	1	0.06
S. paratyphi	2	12,000	100	2,500	100
	24	1,900	15.8	400	16
	48	116	0.97	30	1.2
Sh. sonnei	2	6,800	100	1,200	100
	24	360	5.5	65	5.4
	48	0	0.13	2	0.17
Coxsackie A9*	2	270	100	60	100
	24	32	11.9	9	15
	48	4	1.5	0	<1.67

* The figures given under No. were most probable number of cytopathic units by the method of Chang, *et al.*[5]

Excretion of viable pathogens by the nematodes. In four separate experiments made on both nematodes fed on *S. typhosa*, *S. paratyphi*, *Sh. sonnei*, and Coxsackie A9 virus, not a single bacterial colony nor a single tube showing cytopathic changes was encountered. While the number of worms used in each inoculum was small, the completely negative results suffice to indicate that no viable bacterial cells or virus particles were excreted by these nematodes.

Protection of enteric pathogens by the carrier-nematodes against chlorination. Results in experiments made on both nematodes fed on *S. typhosa*, *S. paratyphi*, *S. typhimurium*, *Sh. sonnei*, and *Sh. dysenteriae* II are summarized in Table 2 and those on the nematodes fed Coxsackie A9 and Echo 7 virus are summarized in Table 3.

Analysis of the contents of these tables reveals several points of interest. First, while the free *Salmonella* and *Shigella* organisms were completely destroyed in 1 minute with as little as 2.5 ppm of residual chlorine, and the free Coxsackie A9 and Echo 7 viruses were completely destroyed somewhere between 1 and 10 minutes (but probably closer to 1 than to 10 minutes) with 2.0–2.2 ppm of residual chlorine, the nematode-borne organisms survived the chlorination process ranging from 120 minutes at 11.8–12.6 ppm to 30 minutes at 83.2–87.5 ppm of residual chlorine.

Second, in a few cases there was a two- to three-fold reduction in the number of organisms or cytopathic units during the chlorination period, suggesting that there might be a slow destruction of the nematode-borne pathogens. But in a majority of the observations the numbers of organisms or cytopathic units recovered were so irregular as to indicate clearly that the differences were due to sampling and experimental errors rather than to the chlorination process.

TABLE 2

Protection of enteric bacteria by carrier nematodes against chlorination in water at pH 6.6–7.2 and 25°C

Pathogen-carrying agent	Chlorine (ppm)		Contact time (min)	(%) motile worms	No. colonies/10 ml supernate or 100 worms				
	Initial	Residual			*S. typhosa*	*S. paratyphi*	*S. typhimurium*	*Sh. sonnei*	*Sh. dysenteriae II*
Supernate	0.0	0.0	—	—	550	940	700	550	450
D. nudicapitatus	(Control)		—	98	7500	11000	9500	9000	3800
Supernate	3.0	2.5	1	—	0	0	0	0	0
		2.3	10	—	0	0	0	0	0
		2.5	1	97	8200	9000	7800	10500	4500
D. nudicapitatus		2.3	10	99	6500	7500	9500	5200	5000
	3.0	2.2	60	96	4500	4300	6000	9000	2000
		1.8	120	95	7000	3200	7500	5800	4700
Supernate	15.0	14.2	1	—	0	0	0	0	0
		14.2	1	99	3500	10500	12000	5100	2800
D. nudicapitatus	15.0	13.8	60	72	4400	4800	5500	9200	3900
		12.6	120	58	6800	7700	8500	6200	2500
Supernate	45.0	43.0	1	—	0	0	0	0	0
		43.0	1	96	2900	5700	4800	8800	2200
D. nudicapitatus	45.0	41.2	30	20	5600	7300	9800	5400	3700
		39.5	60	11	8100	2900	5300	6900	1800
		91.9	5	42	6300	9400	2500	8800	4100
D. nudicapitatus	95.0	88.2	15	16	8900	6200	7100	5100	1500
		83.1	30	3	5900	3200	4900	3100	2000
Supernate	0.0	0.0	—	—	850	1100	450	920	650
C. quadrilabiatus	(Control)		—	96	1900	2100	1200	800	550
Supernate	15.0	14.5	1	—	0	0	0	0	0
		12.7	30	—	0	0	0	0	0
		14.5	1	98	950	1500	1050	1100	750
C. quadrilabiatus	15.0	13.9	30	84	1600	850	480	350	180
		12.7	60	78	1100	570	920	810	350
Supernate	95.0	91.1	5	—	0	0	0	0	0
		91.1	5	48	500	480	750	920	150
C. quadrilabiatus	95.0	88.9	15	22	780	900	270	410	250
		83.2	30	7	380	510	700	580	110

Third, the nematodes showed no mortality in 120 minutes at very low chlorine dosages, namely, 2.5 to 3.0 ppm, or at high dosages in 1 minute contact time.

As contact times increased above 5 minutes with the chlorine dosages of 15 to 100 ppm, there were increasing worm mortality rates. For instance, at 15 ppm initial chlorine there was no loss of motility after a 1-minute contact; but 28 to 31 and 42 to 56% of the *D. nudicapitatus* worms, or 16 to 20 and 22 to 34% of the *C. quadrilabiatus* worms became non-motile after 60 and 120 minutes, respectively. At 95 to 100 ppm of initial chlorine the respective percentages of nonomotile worms of these two species were 58 to 64 and 52 to 57 in 5 minutes, and 84 to 87 and 78 to 81, in 15 minutes contact. The nematode-borne pathogens survived in the worm gut for quite some time after the death of the carrier worms.

TABLE 3

Protection of enteroviruses by carrier nematodes against chlorination in water at pH 6.6–7.2 and 25°C

Virus-carrying agent	Chlorine (ppm)		Contact time (min)	(%) motile worms	Plaque forming unit/10 ml supernate or 100 worms	
	Initial	Residual			Coxsackie A9	Echo 97
Supernate	0.0	0.0	—	—	710	1600
D. nudicapitatus	(Control)		—	99	550	920
Supernate	3.0	2.2	1	—	10	6
		2.0	10	—	0	0
		2.2	1	98	350	840
D. nudicapitatus	3.0	2.0	10	98	600	720
		1.8	60	96	700	950
		1.7	120	95	450	560
Supernate	15.0	14.2	1	—	0	0
		14.2	1	96	650	900
D. nudicapitatus	15.0	12.8	60	59	390	680
		11.8	120	44	500	790
Supernate	50.0	47.6	30	—	0	0
D. nudicapitatus	50.0	47.6	30	28	520	810
		46.0	60	13	480	600
Supernate	100.0	95.6	5	—	0	0
D. nudicapitatus	100.0	95.6	5	36	610	580
		93.1	15	13	490	480
Supernate	0.0	0.0	—	—	1850	1270
C. quadrilabiatus	(Control)		—	98	43	35
Supernate	15.0	14.1	1	—	0	0
		12.8	60	—	0	0
		14.1	1	97	60	44
C. quadrilabiatus	15.0	12.6	60	80	50	30
		11.8	120	66	72	52
C. quadrilabiatus	50.0	44.6	30	48	52	65
		43.1	60	28	40	28
		90.2	5	43	68	54
C. quadrilabiatus	95.0	89.3	15	19	50	33
		87.5	30	5	46	38

Fourth, the *C. quadrilabiatus* appeared to be slightly but definitely more resistant to free chlorine than the *D. nudicapitatus*, since there were consistently higher percentages of nonmotile worms of the latter than of the former species at chlorine dosages of and above 15 ppm and contact times longer than 1 minute. It also seemed that the difference in resistance progressively diminished as the chlorine dosage was increased to 100 ppm.

It is of interest to point out that different stages of development of these nematodes have different degrees of resistance to free chlorine. The nonsheathed larvae are apparently the least resistant form as they were the first to show loss of motility. The adults are more resistant than the nonsheathed larvae, but less resistant than the sheathed larvae which were the last to lose motility during the chlorination process.

DISCUSSION

From the data gathered in the present investigation, it appears that the *D. nudicapitatus* and *C. quadrilabiatus* feed on the *Salmonella* and *Shigella* organisms and the Coxsackie and Echo viruses. The fact that 5–6% of *S. typhosa* or *Sh. sonnei* and 12–16% of *S. paratyphi* or Coxsackie A9 virus survived for 24 hours in the worm gut, and that about 0.1% of the former organisms and about 1% of the latter survived for 48 hours likewise, indicates that these nematodes of sewage-treatment origin may carry some viable pathogens into the surface water that receives the sewage effluent; but their potentiality of serving as pathogen-carrier is quite small.

The high resistance of these nematodes to free chlorine and the even greater protection they offer to the ingested pathogens against chlorination make it plain that if these nematodes carry viable pathogens in their gut at the time of water treatment, chlorination at any degree may not be effective in eliminating the carrier status of the worms.

The longer survival of the *S. paratyphi* and Coxsackie A9 virus than that of the *S. typhosa* and *Sh. sonnei* in the worm gut is an interesting phenomenon. In this connection, it may be pointed out that similar observations were reported by Greenberg[8] on housefly maggots, in which the *S. typhosa* and *Sh. flexneri* were found to disappear more rapidly in the larva gut than the *S. paratyphi*, *S. enteritidis*, and *S. pullorum*. It should also be noted that Melnick and Penner[9] reported the disappearance of the poliovirus in maggots of the bottlefly, *Phaemicia sericata*, 1 to 3 days after they were fed on spinal cord from a fatal case of poliomyelitis and the recovery of only a minimum amount of the mouse-adapted (Lansing strain) of poliovirus 3 days after feeding on mouse cords.

The considerably smaller amount of virus found in the virus-fed nematodes as compared with the amount of bacterial cells in those fed on bacteria was apparently due to the difficulty of these worms feeding on virus particles suspended in fluid. It has been shown in the other report,[2] that these nematodes do not grow well in axenic cultures unless the proteinaceous material is supplied in a coagulated state, in the clumps of which the worms flourish.

As is also shown in the same report,[2] these nematodes appear to depend chiefly, if not entirely, on proteinaceous material for food supply and do not ingest sufficient food to distend the intestine. Their excreta contain predominantly ammonia and carbonate and no visible particulate matter. These observations suggest that the nematodes are capable of digesting a great variety of proteins, including viruses, and explain why no viable microbes were excreted.

SUMMARY AND CONCLUSIONS

The findings gathered in this investigation are summarized as follows:

a) The *D. nudicapitatus* and *C. quadrilabiatus* readily ingested *Salmonella* and *Shigella* organisms on plate cultures and could even ingest small amounts of Coxsackie and Echo viruses in a fluid suspension.

b) About 5 to 6% of the ingested *S. typhosa* or *Sh. sonnei* and about 12 to 16% of the ingested *S. paratyphi* or Coxsackie A9 virus survived for 24 hours; but the survivals of these were reduced to about 0.1% and 1% respectively after 48 hours. There was no evidence of excretion of viable pathogens.

c) These nematodes are so highly resistant to the destructive action of free chlorine in water that they were not affected by 2.5–3.0 ppm of chlorine in a 120-minute exposure or by 15 to 45 ppm of chlorine in a 1-minute exposure when the water temperature was 25° C, pH 6.6–7.2, and the chlorine residuals were only slightly lower than initials. Even with an initial chlorine as high as 95–100 ppm, 50–60% of these nematodes survived a 5-minute contact and 10–20% survived a 15-minute contact. The *C. quadrilabiatus* appeared to be somewhat more resistant than the *D. nudicapitatus*, but the difference was reduced as the chlorine dosage was increased from 15 to 95–100 ppm.

d) The ingested pathogens were protected by the carrier nematodes to the extent that they had a complete survival even when about 90% of the worms were immobilized by the free chlorine.

It is concluded that nematodes of the Rhabditidae family and of sewage-treatment origin may be potential carriers of human enteric bacteria and viruses. Although a majority of the ingested pathogens may disappear in 1 to 2 days after ingestion, 5 to 16% may remain viable if the worms are carried to the water

supply within 24 hours after departure from their habitat and will survive routine chlorination as practiced in the field.

ACKNOWLEDGMENT

The authors acknowledge the help of Mr. Bernard A. Kenner in conducting the fermentation and infrared spectrophotometric tests in confirming *Salmonella* and *Shigella* species.

REFERENCES

1. Chang, S. L., Austin, J. H., Poston, H. W., and Woodward, R. L., 1959. Occurrence of a nematode worm in a city water supply. *J. Am. Water Works A.*, **51**: 671–676.
2. Chang, S. L. Cultivation and life cycle of *Rhabditis limicola*, *Diplogaster nudicapitatus*, and *Cheilobus quadrilabiatus*. To be published.
3. American Public Health Association, American Water Works Association, and Federation of Sewage and Industrial Wastes Associations, 1955. *Standard Methods for Examination of Water, Sewage, and Industrial Wastes*, p. 137, 10th ed., American Public Health Association, Inc., New York, N. Y.
4. Chang, S. L., 1958. Cultural, cytological and ecological observations on the amoeba stage of *Naegleria gruberi*, *J. Gen. Microbiol.*, **18**: 565–578.
5. Chang, S. L., Berg, G., Busch, K. A., Stevenson, R. E., Clarke, N. A. and Kabler, P. W., 1958. Application of the "Most Probable Number" method for estimating concentrations of animal viruses by the tissue culture technique. *Virology*, **6**: 27–42.
6. Dulbecco, R., 1952. Production of plaques in monolayer tissue cultures by single particles of virus. *Proc. Natl. Acad. Sci. U. S.*, **38**: 747–752.
7. Hsiung, G. D. and Melnick, J. L., 1957. Morphologic characteristics of plaques produced in monkey kidney monolayer cultures by enteric viruses (poliomyelitis, Coxsackie, and Echo groups). *J. Immunol.*, **78**: 128–136.
8. Greenberg, B., 1959. Persistence of bacteria in the developmental stages of the housefly. *Am. J. Trop. Med. & Hyg.*, **8**: 405–411.
9. Melnick, J. L. and Penner, L. R., 1952. The survival of poliomyelitis and Coxsackie viruses following their ingestion by flies. *J. Exper. Med.*, **96**: 255–271.

PHYCOMYCOSIS (MUCORMYCOSIS) IN INDONESIA—DESCRIPTION OF A CASE AFFECTING THE SUBCUTANEOUS TISSUE

LIE KIAN JOE*, NJO-INJO TJOEI ENG*, SUTOMO TJOKRONEGORO† AND CHESTER W. EMMONS‡

Only a few of the fungi which cause disease in man fall within the Class *Phycomycetes*. The most important of these pathogens is *Coccidioides immitis* which occupies an uncertain and anomalous taxonomic position. Other pathogenic Phycomycetes are species of *Absidia* and *Rhizopus* belonging to the family *Mucoraceae* and a species of *Basidiobolus* of the family *Entomophthoraceae*. In tissue sections the fungi representing these two families cannot be differentiated with certainty. All are characterized by branching hyphae of large diameter and infrequent septation. Specific identification requires a study of the fungus in culture.

The name phycomycosis is used to designate a fungus infection caused by a member of the *Phycomycetes*. Its use was proposed by Lie Kian Joe *et al.*[1] in order to avoid the more restricted connotations of mucormycosis, while the name of coccidioidomycosis is retained.

Phycomycosis is rare, although found in increasing frequency in certain countries. It was reported until recently only from temperate climates and therefore not called to the attention of workers in tropical countries. However, the disease may occur more frequently in these countries than the lack of reported cases would suggest. Since 1956 six cases of phycomycosis have been found in Indonesia of which four have been reported. One presented an infection of the stomach wall[2] associated with the presence of a large ulcer. No culture of the fungus was obtained and the patient recovered after partial gastrectomy. Two unusual cases, occurring in children, presented an infection of the subcutaneous tissue, causing an extensive, diffuse swelling which covered a large area of the body.[3, 4] Both cases healed spontaneously. A species of *Basidiobolus*, identified as *B. ranarum*, was isolated from one of these patients. Drechsler[5] who has studied this fungus states that it differs from *B. ranarum* as described by Eidam. This fungus is a member of the family *Entomophthoraceae*. It is saprophytic and found in many parts of the world in the digestive tract of frogs and lizards and in beetles which feed upon the excrement of these animals and are in turn eaten by them. The fourth case was a fatal infection of the brain[1] associated with diabetes mellitus. The fungus was not isolated from this case.

Recently, two other cases of infection with a phycomycete in children, causing an extensive swelling of the subcutaneous tissue, have been found. This paper deals with one of the cases while the other will be published later.

CASE REPORT

An Indonesian girl, age 7 years, was admitted to the General Hospital in Bengkulu, South Sumatra, on January 28, 1957. She showed an extensive, diffuse, subcutaneous swelling, covering the right upper arm extending from just above the elbow to the axilla, the lateral scapular region and the deltoid region (Figures 1 and 2). The infection started as a subcutaneous nodule on the upper arm several months before admission to the hospital. No history of a preexisting insect bite or thorn injury could be obtained. The swelling was sharply demarcated, firm, approximately 1 cm thick in the upper arm with thinner edges, and was about 3 cm thick in the post axillar region. The swelling was smooth, as far as could be judged by palpation through the overlying, somewhat edematous, but intact skin. It was not painful. Movement of the arm was somewhat restricted because of the large swelling in the axillar and deltoid regions.

The patient felt healthy. Her general condition was good. She went to school daily despite her swollen arm. There was no fever. Hemoglobin value was 60%. Differential count: eosinophils 4%, band form neutrophils 9%, segmented

* Department of Parasitology and General Pathology, School of Medicine, Djakarta.
† Department of Pathology, School of Medicine, Bajakarta, Indonesia.
‡ U. S. Department of Health, Education and Welfare, Public Health Service, National Institute of Allergy and Infectious Diseases, Laboratory of Infectious Diseases, National Institutes of Health, Bethesda 14, Maryland.

FIG. 1. Subcutaneous swelling of the right upper arm and deltoid region.

FIG. 2. Subcutaneous swelling of the right upper arm and scapular region. The wound on the dorsal part of the upper arm is the place of the first biopsy. The second biopsy specimen was taken from the post-axillar swelling.

neutrophils 63%, lymphocytes 22% and monocytes 2%.

HISTOPATHOLOGY

On January 29, 1957 a biopsy was performed. A piece of tissue measuring 1.5 cm x 1 cm x 0.5 cm was taken from the swelling in the dorsal part of the upper arm, about 8 cm above the elbow (Figure 2).

Microscopic examination revealed granulomatous tissue, in which cross and longitudinal sections of large branching hyphae with infrequent septations were seen. The diameter of the hyphae varied from 5.4 μ to 18.5 μ. The walls were usually thin and dark blue in color in the hematoxylin-eosin stained slides. Most of the hyphae appeared to be empty probably because the contents had fallen out during the process of mounting and staining, or because extreme shrinkage of the vacuolate cytoplasm had occurred. Some hyphae contained a light blue or light purple staining granular cytoplasm. The hyphae were not numerous; only a few were found in each slide. Since they occurred singly and had a thin wall, the walls were not easily seen in the hematoxylin-eosin stained slides. However, most of the hyphae were surrounded by a band of granular eosinophilic material. These eosinophilic bands measuring in width from 3 μ to 15 μ stood out clearly in the histological slides and were detected easily under the low power. When examined under the high power, the thin wall of the usually empty hypha could be seen adhering to the inner surface of the eosinophilic ring (Figures 3–6).

The granulomatous tissue was characterized by the presence of numerous eosinophils and

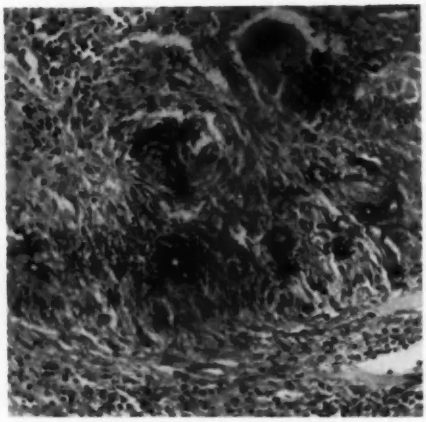


FIG. 3. Granulomatous tissue with giant cells. Hyphae (see arrows) are surrounded by a ring of eosinophilic material and necrotic tissue. Second biopsy specimen. Hematoxylin and eosin stain. × 105.

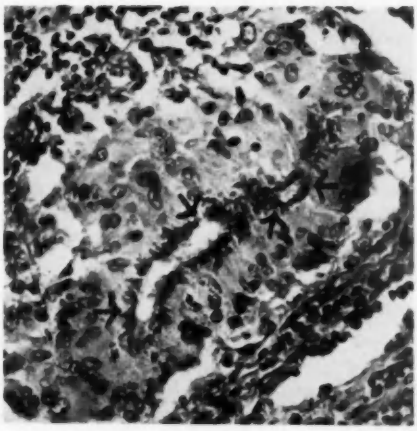


FIG. 4. Hypha (see arrows) with eosinophilic material, enclosed by giant cells. Second biopsy specimen. Hematoxylin and eosin stain. × 280.

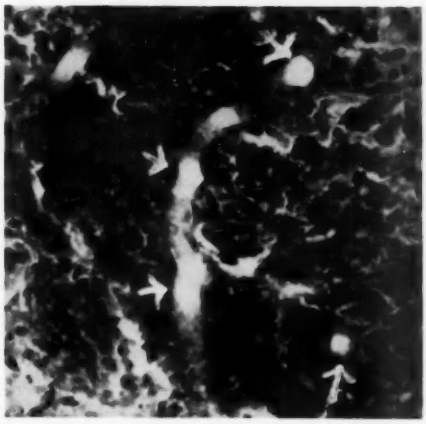


FIG. 5. Empty hyphae (see arrows) in necrotic tissue. Second biopsy specimen. Hematoxylin and eosin stain. × 396.

pseudotubercles with foreign-body giant cells. Eosinophils were found throughout the whole granulomatous tissue. In certain areas they were present in such large numbers that the stained tissue appeared red. Small necrotic areas and abscesses containing eosinophils and neutrophils were also found usually in association with the presence of hyphae. In other areas fibrous tissue and fibroblasts predominated. The blood vessels showed no abnormality. Hyphae were found in the pseudotubercles, in foreign-body giant cells, in necrotic areas, in abscesses and also in other areas of the granulomatous tissue.

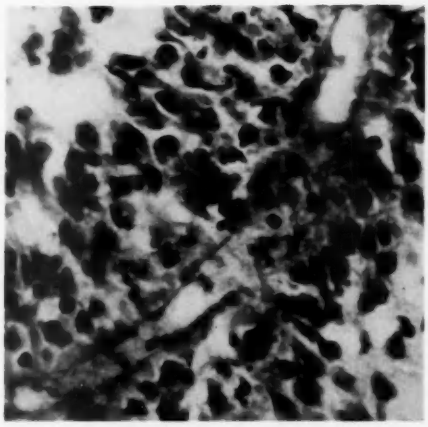


FIG. 6. Hypha in granulomatous tissue. Eosinophilic material is present along the hypha. Second biopsy specimen. Hematoxylin and eosin stain. × 504.

CULTIVATION OF THE FUNGUS

A piece of tissue 8 cm long, 5 cm wide and 3 cm thick was taken from the post-axillar swelling on February 22, 1957 for culture and further histological study.

This piece of tissue was easily loosened from the corium and from the underlying tissue. The swelling was apparently confined mainly to the subcutaneous fat tissue. The tissue was firm with a white-gray color and with small yellow dots on the cut surface. Small pieces of this tissue were inoculated on 20 tubes of Sabouraud glucose agar and on 20 tubes of Littman oxgall agar. The inoculation was done in the operating room immediately after removal of the tissue with adequate precautions to prevent contaminations. The remaining tissue was fixed in formalin. Within 3 days 21 colonies of a rapidly growing fungus were found in 17 Sabouraud glucose-agar slants. The 3 other Sabouraud agar tubes were contaminated with different fungi while the Littman oxgall agar remained negative.

Microscopic examination (Figures 3–6) revealed the same eosinophilic granulomatous tissue as found in the first biopsy specimen. More hyphae were seen than in the first biopsy specimen and there was more necrosis, and there were more foreign-body giant-cells. These giant cells were seen enclosing large portions of hyphae. Abscess formation was less marked. The granulomatous tissue seemed not to invade the corium of the skin. In one area there was a superficial invasion of the underlying muscle tissue show-

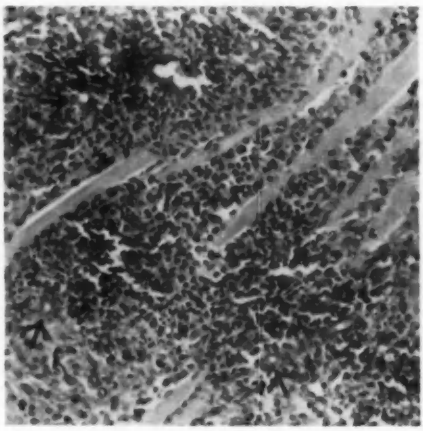


FIG. 7. Necrotic muscle tissue with hyphae (see arrows) and infiltrated with numerous eosinophils and mononuclear cells. Second biopsy specimen. Hematoxylin and eosin stain. × 105.

ing widespread necrosis (Figure 7), but the main part of the removed granulomatous tissue was situated outside the superficial fascia.

FURTHER COURSE OF THE DISEASE

The biopsy operation wound soon healed and the patient was discharged at the parent's persistent request.

On April 17, 1957 the patient returned to the hospital. The swelling had increased in size. The skin overlying the swelling was edematous and bluish-red in color. Unfortunately, permission could not be obtained for a second biopsy, and a second isolation of the fungus could therefore not be made. The patient left the hospital in fear of a second operation.

In December 1957 we visited the patient in her village. We were surprised to find her almost recovered from her disease. There was no swelling in the upper arm. The subcutaneous tissue felt only somewhat tough. Apparently, the disease healed without treatment.

MYCOLOGIC STUDY

The fungus produced in culture on modified Sabouraud's agar, a gray to yellowish-gray colony with a coarse mycelium, principally on the surface of the medium but with some aerial hyphae. Colonies did not grow uniformly either in rate or type of growth and most colonies bore segments of either reduced or increased mycelium production.

Growth was slower than that of most Phycomycetes. Colonies reached a diameter of 5 cm in 8–10 days and usually did not exceed this diameter, even after continued incubation. A temperature of 37°C inhibited growth.

The mycelium was coenocytic and few septa were found in the hyphae. The hyphae were broad and the width varied greatly and abruptly so that occasionally diameters of 15 or 20 μ were observed. Branches were very irregular in location, size and incident angle.

All attempts to induce sporulation failed in our laboratories and in the laboratory of an able specialist in the Phycomycetes. No spores of any kind were observed and the fungus could not be identified. Its appearance in tissue is compatible with that of a species of *Mortierella* isolated from an unpublished case seen by the junior author and the type of growth seen in culture would not exclude the fungus from this genus. The evidence is too tenuous, however, to identify the Indonesian fungus with this genus.

The coenocytic nature of the fungus, the extreme size and fragility of its hyphae and lack of sporulation made continued subculture difficult. Unfortunately, all strains of the fungus died before its study was completed.

DISCUSSION

This case was strikingly similar to the two cases of subcutaneous phycomycosis described previously in two Indonesian children.[3, 4] In all three cases the following characteristic features were found:

1. The disease affected the subcutaneous tissue, mainly the fat tissue associated with the formation of a granuloma with pseudotubercles covering a large area of the body and characterized by the presence of large numbers of eosinophils. The skin covering the lesion was intact.

2. Large, branching, coenocytic hyphae were present throughout the active granulomata.

3. The disease occurred in children and healed spontaneously.

From the first case a fungus, identified as *Basidiobolus ranarum*, was isolated in culture. The fungus from the second case was not isolated. It is interesting that *Basidiobolus* was not isolated from the present case, but another Phycomycete. It is unfortunate that species diagnosis of the isolated fungus has not been possible because spores have not been obtained

in culture. It is assumed that the isolated fungus was not a contaminant since isolation was made immediately after removal of the tissue with adequate precautions to prevent contamination and since it was found in pure culture in most of the inoculated Sabouraud media. Apparently, different fungi belonging to the class *Phycomycetes* can cause this distinctive and characteristic lesion.

Close histological comparison with the case from which *B. ranarum* was isolated revealed no important differences. There were more hyphae found in the histological slides of the second biopsy specimen. They were probably responsible for the more widespread tissue necrosis and more marked giant cell formation. Also, there was invasion of the muscle tissue which was not found in the two previous cases. However, these differences cannot be considered of much significance. Probably much depends on the area where the biopsy specimen was taken as well as on the stage of development of the lesion.

These three cases and also another case found recently, which will be published later, present a characteristic disease entity. The localized, painless, subcutaneous swelling, covered with intact skin, occupying a large area of the body, and lasting for many months is so typical that one would suspect the disease from clinical examination only. The diagnosis can be confirmed by finding large, branching, coenocytic hyphae in the tissue sections. When these hyphae are not scarce, such as in the second biopsy of this case, they are easy to find. However, they are usually scarce. Moreover, they have usually a thin wall which makes them often difficult to find in the hematoxylin-eosin stained slides. In addition, hyphae are often damaged, probably by the action of leucocytes and tissue cells, and this also makes recognition more difficult. However, the presence of a granuloma with numerous eosinophils is a good clue for making a serious attempt at finding them. Staining with the periodic acid-Schiff stain is often helpful. In one of the observed cases, only after making numerous slides were the hyphae found. The band of eosinophilic granular material surrounding many of the hyphae like a broad ring is useful for finding the hyphae. These structures stand out clearly in the hematoxylin-eosin stained slides. When observed under the high power, the thin, dark-blue wall of the usually-empty hypha can often be seen attached to the inner surface of the eosinophilic ring. Species diagnosis of the fungus causing the disease can only be made by studying it in culture.

It is not known how infection takes place in all these cases. The disease starts as a small nodule under the intact skin. No insect bite or injury by a thorn or splinter was known to precede lesions.

A few cases of phycomycosis, mainly from European countries, were reported before 1943. This literature has been summarized by Gregory et al.[6] Most of the cases observed before 1943 were of pulmonary infection, although cases affecting the ears and gastric wall were reported. Infections of the nose and skin were quite uncommon and the central nervous system was least affected. Generalized dissemination of the fungus was observed once.

Since 1943 reports of phycomycosis are increasing in frequency. The disease has been reported mainly from the U. S. A., but also from England and Canada. Baker,[7] who summarized the literature since 1943, collected 28 cases including his own observations. The disease as observed since 1943 has the following features. It takes mainly cerebral and also pulmonary forms and, rarely, intestinal, ocular and disseminated forms. The cerebral forms are usually associated with orbital and paranasal sinus infection. It occurs at all ages. It is acute rather than chronic and is usually fatal, although recovery in two patients has been observed.[7, 8] The disease is usually found as a complication of other diseases, of which uncontrolled diabetes is the commonest, but other diseases, such as leukemia, multiple myeloma, Hodgkin's disease, fatal burns, uremia, cirrhosis and diarrhea are also antecedent conditions. Cortisone, corticotropin, anti-leukemic chemical agents and antibiotics may also be predisposing factors.[7] The spread of the fungus is unique in that it has a great tendency to invade the tough arterial and venous walls, causing thrombosis. The fungus has been isolated from three cases and was found to belong to the genus *Rhizopus*.[7, 8, 9]

Not included in Baker's publication were 19 recently reported cases, including the 4 cases from Indonesia. The cases observed outside Indonesia were similar to those summarized by Baker[7] in that they occurred as a complication of other diseases, while the fungus grew readily into the blood vessels. Thirteen of these cases

were found in the U. S. A., eight presenting an infection of the lungs,[10, 11, 12] four of the brain[10, 13] and one of the skin.[14] One case was reported from Canada[15] and one from Australia,[16] both affecting the brain. The case of skin phycomycosis healed after treatment with potassium iodide, while the other 14 died either because of the primary disease, the fungus infection or because of both.

Subcutaneous phycomycosis as reported here has been found until now only in Indonesia. It is unique in that it differs in many respects from phycomycosis described in other countries. It is found up to now only in children; there is no predisposing factor; it is chronic and heals spontaneously; it produces an eosinophilic granuloma which is not found in other human phycomycosis; the fungus has no tendency to grow into blood vessels, and a *Basidiobolus* species and an unidentified phycomycete have been isolated.

It seems that subcutaneous phycomycosis is not very rare in Indonesia. It is likely that the disease will be found more frequently with growing awareness of it among physicians. It is possible that it also occurs in other tropical countries.

SUMMARY

We report a case of subcutaneous phycomycosis occurring in an Indonesian girl 7 years old. It is the third case reported from Indonesia. The fungus, a phycomycete, was isolated, but species diagnosis was not possible because all attempts to induce sporulation failed.

REFERENCES

1. Lie-Kian-Joe, Njo-Injo Tjoei Eng, Sutomo Tjokronegoro, Schaafma, Sj., and Emmons, C. W., 1959. Phycomycosis of the central nervous system associated with diabetes mellitus in Indonesia. *Am. J. Clin. Path.*, **32:** 62–70.
2. Lie-Kian-Joe and Njo-Injo Tjoei Eng, 1956. A case of mycosis of the stomach caused by a phycomycete. *Doc. Med. Geogr. Morb. Trop.*, **8:** 249–252.
3. Lie-Kian-Joe, Njo-Injo Tjoei Eng, Pohan, A., van der Meulen, H., and Emmons, C. W., 1956. *Basidiobolus ranarum* as a cause of subcutaneous mycosis in Indonesia. *A.M.A. Arch. Dermat.*, **74:** 378–383.
4. Emmons, C. W., Lie-Kian-Joe, Njo-Injo Tjoei Eng, Pohan, A., Kertopati, S., and van der Meulen, A., 1957. *Basidiobolus* and *Cercospora* from human infections. *Mycologia*, **49:** 1–10.
5. Drechsler, C., 1958. Formation of sporangia from conidia and hyphal segments in an Indonesian *Basidiobolus*. *Am. J. Botany*, **45:** 632–638.
6. Gregory, J. E., Golden, A., and Haymaker, M., 1943. Mucormycosis of the central nervous system. Report of three cases. *Bull. Johns Hopkins Hosp.*, **73:** 405–419.
7. Baker, R. D., 1957. Mucormycosis—A new disease? *J.A.M.A.*, **163:** 805–808.
8. Harris, J. S., 1955. Mucormycosis. Report of a case. *Pediatrics.*, **16:** 857–867.
9. Bauer, H., Ajello, L., Adams, E., and Hernandez, U., 1955. Cerebral mucormycosis. Pathogenesis of the disease. *Am. J. Med.*, **18:** 822–831.
10. Keye, J. D., and Magee, W. E., 1956. Fungus diseases in a general hospital. A study of 88 patients. *Am. J. Clin. Path.*, **26:** 1235–1253.
11. Mayfield, G. R., and Condie, F., 1957. Paradoxical mucorthrombosis in thrombocytopenic purpura. *A.M.A. Arch. Path.*, **63:** 260–264.
12. Stefanini, M., and Allegra, S., 1957. Pulmonary mucormycosis in acute histiocytic leukemia. *New Eng. J. Med.*, **256:** 1026–1030.
13. Smith, M. E., Burnham, D. K., and Black, M. B., 1958. Cerebral mucormycosis. Report of a case. *A.M.A. Arch. Path.*, **66:** 468–473.
14. Jackson, J. R. and Karnauchow P. N., 1957. Mucormycosis of the central nervous system. *Canad. Med. Ass. J.*, **76:** 130.
15. Josefiak, E. J., Foushee, J. H. S., and Smith, L. C., 1958. Cutaneous mucormycosis. *Am. J. Clin. Path.*, **30:** 547–552.
16. Clinicopathological Conference, Sydney Hospital, 1956. *Med. J. Australia*, **2:** 30–34.

A COMBINATION OF AMODIAQUIN AND PRIMAQUINE (CAMOPRIM) IN THE PREVENTION AND CURE OF SPOROZOITE-INDUCED CHESSON STRAIN VIVAX MALARIA

KENNETH O. COURTNEY,* ROBERT HODGKINSON,* REX RAMSEY,† AND MEIGS HAGGERTY*

Until recently those concerned with malaria control and eradication have depended almost entirely on spraying of residual insecticides within human habitation and occasionally in adjacent structures. In those areas where the anopheline mosquitoes bit man indoors and rested indoors and where the insecticide could be applied cheaply the procedure was relatively effective. However, it soon became apparent that certain areas were not suited to this procedure and that transmission of malaria could not be effectively interrupted by it. There are malarious areas where the use of residual insecticides is impractical if not impossible because the mosquito vector is an outdoor biter and rester, or due to the sparseness and/or the nomadic habits of the populations, or because of lack of transport and communications. Further, there is an increasing number of malarious countries where the vector has acquired resistance to one or all safely-usable insecticides.

Such considerations have stimulated the suggestion that mass chemotherapy be utilized as a substitute in those areas where residual insecticides could not be used effectively. Such a program would produce a chemical barrier against patent malaria and against transmission of the infection. This would require a drug that is effective, inexpensive, acceptable to the people, nontoxic on continued use and practical in application to large populations.

Edgcomb *et al.*[1] showed that primaquine was a curative agent against the Chesson strain *vivax* malaria. Such treatment was equally effective against Korean *vivax* when administered at the time of an acute attack, Alving *et al.*,[2] or during long-term latency, Coatney *et al.*[3] Arnold *et al.*[4] have shown the effectiveness of this treatment against the blood and tissue stages of *P. falciparum*. The foregoing effects were achieved by giving primaquine daily; Arnold *et al.*,[5] and Alving and Brucker[6] subsequently reported that such combinations of drugs were curative when primaquine was given intermittently. Jeffrey *et al.*[7] have shown that a single dose of primaquine quickly eliminates infective gametocytes in *falciparum* infections.

The effectiveness of amodiaquin (Camoquin®) as a suppressive agent has been studied in Brazil by Pendio *et al.*[8] and Mein *et al.*,[9] in Egypt by Halawani *et al.*,[10, 11] in India by Simeons and Chhatre,[12] in the United States by Coatney *et al.*[13] and many others. All of these investigators pointed to its being an active antimalarial of low toxicity.

Walker[14] pointed out the potentialities of monthly doses of amodiaquin plus a gametocidal drug for malaria control and, in the same year, Mitra and Chaudhuri[15] confirmed the suppressive action of combined amodiaquin and primaquine.

In view of these properties of amodiaquin and primaquine and in consideration of the possible importance of the use of these drugs, in combination, for malaria eradication in areas where residual insecticides cannot be used effectively, it was decided to do a carefully controlled evaluation of a formulation consisting of amodiaquin and primaquine.

The resulting study was designed to evaluate this drug combination with respect to (1) the prevention of patent infections when given to the individual before and after being bitten by infected mosquitoes and (2) the radical cure (without relapse) of the infection in patients with clinical malaria when the drug combination was given weekly or bi-weekly. An additional facet of the study was to evaluate the effects of amodiaquin on mosquito infectivity.

MATERIALS AND METHODS

This study was carried out at the Florida State Prison in Raiford, Florida, where 18 subjects were utilized. They were white, male, inmate volunteers, 20 to 40 years of age, in good physical condition and with no previous history of malaria.

* Department of Clinical Investigation; Parke, Davis & Company.
† Chief Medical Officer, Florida State Prison, Raiford, Florida.

These volunteers were made fully aware of the nature of the trials.

The group of 18 volunteers was divided by lottery drawing into three sub-groups of six each. Group A was used for evaluating the ability of the drug combination to prevent patent infection. Groups B and D served for evaluation of the radical curative effects after infection had been allowed to develop and when treatment was given weekly and bi-weekly, respectively. (Group C designates the patients used only for plasmodia strain maintenance.)

Each volunteer was bitten by *Anopheles quadrimaculatus* mosquitoes infected with the Chesson strain of *Plasmodium vivax*. The Chesson strain relapse pattern is consistent with fairly regular and repeated re-invasions of the blood stream from a fixed-tissue site,[16] and for this reason it was selected for these trials. The mosquitoes obtained their infection by feeding on reservoir volunteers (Group C), infected with the same strain, approximately 2 weeks earlier.

Infection was accomplished by allowing a mosquito to bite 2 volunteers, correspondingly numbered in groups A and B, by a technique of interrupted feeding. Group B served as the control for group A in which treatment was started prior to infection by bites of infected mosquitoes. After feeding on the second subject, the mosquito was dissected and infection determined by the presence or absence of sporozoites in the salivary glands. The same method was followed in group D except cross-biting was done between pairs within the group. Each volunteer was bitten and re-bitten until he had received 10 positive bites. Observations on the volunteers included 4-hour temperature readings and thick and thin blood films for parasitemia daily from the 10th day after mosquito bites, through the febrile period and until two negative smears appeared following treatment of the acute attack. At least 100 thick film fields were examined before a smear was considered negative. Additional blood smears were made and examined weekly during the next 20 weeks in groups A and B and for 30 weeks in group D.

The formulation of amodiaquin and primaquine is to be referred to as "Camoprim."* Each Camoprim tablet contains amodiaquin as the dihydrochloride salt and primaquine as the diphosphate salt in the quantity of 150 mg amodiaquin base and 15 mg primaquine base.

* Camoprim is the trademark of Parke, Davis & Company for the combination of amodiaquin and primaquine.

TESTS AND RESULTS

Evaluation of Camoprim in the prevention of patent infections. It was originally planned to give each volunteer of Group A the first dose† of Camoprim, 2 days prior to infection by mosquito bites and then to repeat this dose once weekly for a total of 10 weeks. However, an unfortunate accident in the insectary after the patients had been placed on the drug made it necessary to postpone infection as planned consequently each volunteer received 8 single weekly doses prior to infection. This dose was repeated weekly for 10 weeks after infection.

Of the six volunteers in this test, none developed fever and no parasites were found during an observation period of over 6 months. As a further check to determine whether the group A patients harbored infection separate lots of 10 non-infected mosquitoes were allowed to feed on each of these subjects on days 20, 22 and 24 after being bitten by infected mosquitoes. None of the mosquitoes became infected.

Evaluation of radical curative effects of Camoprim. The chief purpose of this phase of the study, using the groups B and D patients, was to determine whether or not small doses (two tablets) of Camoprim would eradicate established infections when given weekly or fortnightly, respectively. Such Camoprim doses deliver only 300 mg of amodiaquin base, which were considered too small for rapid control of the early clinical attacks in non-immune white patients. Hence, it was decided at the outset that amodiaquin alone (600 mg of base) would be used for control of the acute attack and that Camoprim would be started 72 hours later. This plan enabled concomitant observations on the effects of amodiaquin on mosquito infectivity, which are described in a separate section of the report.

Five of the 6 group B patients developed patent infections within 15 to 19 days after they were bitten by infected mosquitoes; one subject (B 1) failed to show evidence of infection through an observation period of 29 days. Among the 5 that became infected, 4 had positive blood smears by the 15th day and developed severe clinical

† In this study "a dose" will be considered to consist of 2 tablets of Camoprim (a total of 300 mg amodiaquin base and 30 mg primaquine base).

malaria by the 17th day. The pertinent data on these 5 patients are shown in Figure 1. One patient (B 6) showed parasites first on the 19th day but because of extraneous circumstances, he was not allowed sufficient time to develop clinical malaria. All 6 patients were treated successively with amodiaquin and Camoprim. A single dose of amodiaquin (600 mg of base) given on the 20th day after infection, stopped the chills and fever within one day and caused the disappearance of parasites from the blood stream within 24 to 36 hours. Weekly doses of Camoprim were started on the 24th post-infection day and were continued for 10 weeks. None of the weekly blood smears was positive for parasites and none of the patients had any clinical evidence of malaria from the time Camoprim was started and on through a total observation period of 150 days following the last dose of Camoprim.

Group D. The trials in the Group D patients conformed to the same pattern as those of Group B except that Camoprim was given every 2 weeks instead of weekly. One volunteer withdrew from the study at the outset. Four of the 5 volunteers developed parasitemia and severe clinical malaria 15 to 16 days after being bitten by infected mosquitoes. Subject D 6 failed to exhibit evidence of infection within 20 days even though his "biting partner" D 5 developed clinical malaria on day 16. Circumstances precluded awaiting his possible subsequent development of malaria and he was treated along with the other group D patients. It was recognized that the absence of subsequent malaria in this patient would be inconclusive. Treatment of the patients with a single 600 mg (base) dose of amodiaquin on the 20th post-infection day was rapidly effective (Figure 2). Camoprim was started on the 24th post-infection day and was continued every 2 weeks for a total of 10 doses (20 weeks). None of the weekly blood smears was positive for parasites and none of the patients had any clinical evidence

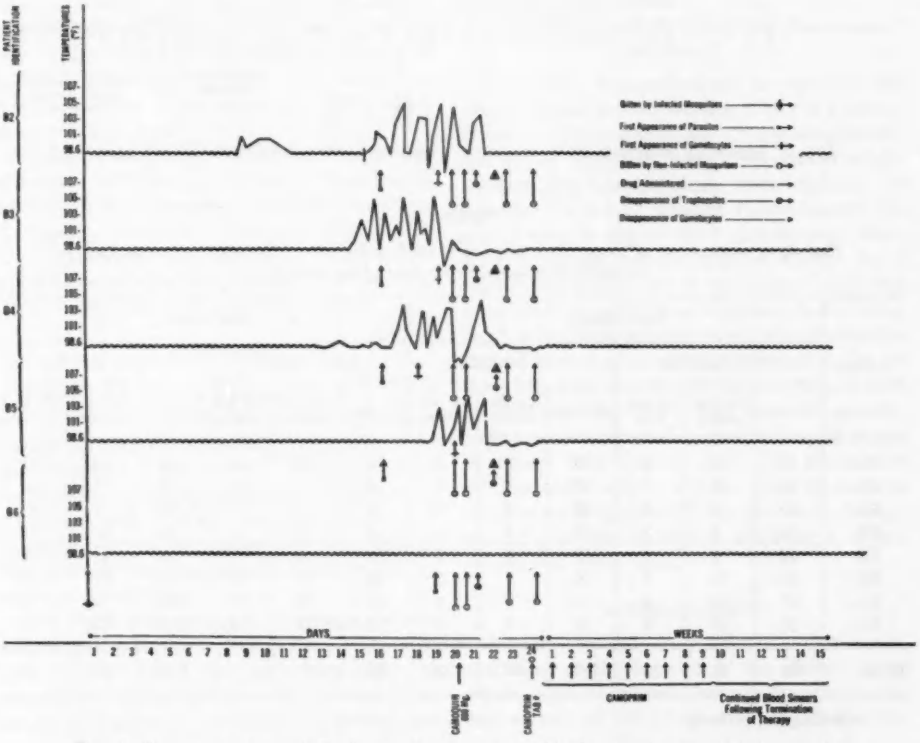


FIG. 1. Summary of events in 5 volunteers of group B who developed clinical malaria

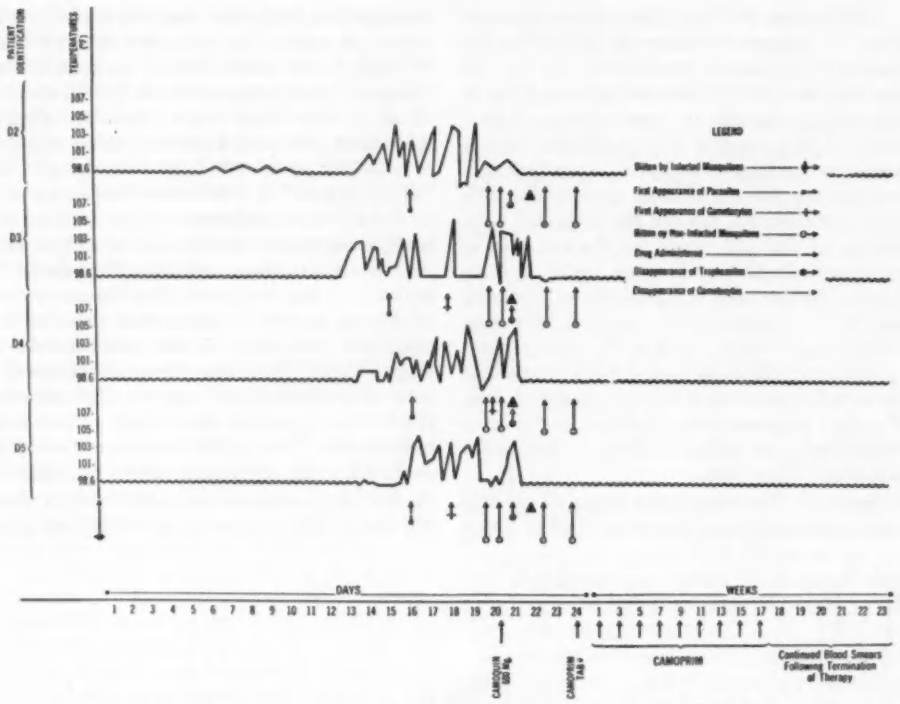


FIG. 2. Summary of events in 4 volunteers of group D who developed clinical malaria

TABLE 1

Results of dissections of mosquitoes allowed to feed on infected volunteers before and after receiving a single 600 mg dose of amodiaquin

Patient	Before therapy						After therapy*					
	12 hrs. before Rx			1 hr. before Rx			36 hrs. after Rx			72 hrs. after Rx		
	No. fed	No. dissected	No. positive	No. fed	No. dissected	No. positive	No. fed	No. dissected	No. positive	No. fed	No. dissected	No. positive
B2	10	5	0	10	3	3	10	9	0	15	12	0
B3	10	5	1	10	7	2	10	1	0	10	7	0
B4	10	4	2	10	5	4	10	2	0	10	7	0
B5	10	7	1	10	7	1	10	5	0	20	18	0
D2	10	5	0	10	6	2	10	3	1	15	11	0
D3	10	10	3	10	4	2	10	1	0	15	11	0
D4	10	10	3	10	7	4	10	5	0	10	9	0
D5	10	10	3	10	9	4	10	5	0	15	11	0
Total . . .	80	56	13	80	48	22	80	31	1	110	86	0

* Amodiaquin, 600 mg.

of malaria from the time Camoprim was started and on through a total observation period of 120 days following the last dose of Camoprim.

Effects of Amodiaquin on mosquito infectivity. This portion of the study was done in groups B and D during their acute attack of malaria and *before* Camoprim therapy. Mature gametocytes appeared in the blood of 8 volunteers in group B and D on the 19th post-infection day.

In order to ascertain whether each subject was infective to mosquitoes a single pretreatment test lot of non-infected mosquitoes was allowed to feed on each volunteer. Eleven hours later a similar biting episode was accomplished with new lots of mosquitoes (Table 1). Immediately following the second biting period, each subject was given 600 mg base of amodiaquin. At 36 and 72 hours post-therapy, separate lots of non-infected mosquitoes were allowed to feed until satiated on each volunteer. The 104 pretreatment mosquitoes dissected 7 days after biting, showed overall infection rates of 22% and 46% at 12 hours and 1 hour respectively prior to therapy. The higher infection rate at the later time was correlated with visible evidence of a larger percentage of mature gametocytes. As mentioned earlier, all visible parasites disappeared from the blood within 24 to 36 hours.

Of the 80 mosquitoes which were fed on the volunteers 36 hours following amodiaquin therapy, 31 were dissected 7 days later and only one showed infection (1 oöcyst). Of the 110 mosquitoes which were fed on the volunteers 72 hours following amodiaquin therapy, 86 were dissected 7 days later and none was infected.

DISCUSSION

Ideally for a drug or combination of drugs to serve optimally in a malaria eradication program, it should provide the following (1) protection from manifestations of the disease, (2) interruption of transmission of malarial parasites to the mosquito population and (3) cure of residual infections in people so they will not relapse after the period of mass therapy and thus serve as a reservoir of infection to mosquitoes.

The result with group A volunteers demonstrated that, when given weekly for 8 weeks prior to and for 10 weeks after they were bitten by mosquitoes heavily infected with *P. vivax*, Chesson strain, Camoprim prevented the development of any evidence of malaria. Whether fewer doses would have produced the same effect is not known, but since the use of drugs in an eradication program would of necessity extend over a much longer period, it is of little practical importance. Further, there is no evidence to show that other types of malaria would be more refractory to such preventive therapy than the Chesson strain of *P. vivax*.

With reference to preventing infection of mosquitoes, this study demonstrated that even amodiaquin alone was effective in *P. vivax* infections, while Jeffrey *et al.*,[7] have shown the pronounced action of primaquine alone against *P. falciparum* infections.

The efficacy of Camoprim in curing latent infections and in preventing relapses was borne out by the results because, when this preparation was given at a single dose once weekly for 10 weeks (group B) and at a single dose every other week for 20 weeks (group D), no relapses developed or other evidence of infection appeared during an observation of 6 months.

SUMMARY

This study was undertaken to evaluate the protective and radical curative effects of a preparation in tablets containing 150 mg amodiaquin and 15 mg primaquin when used against sporozoite-induced Chesson-strain *vivax* malaria. It was effective in both respects. Patent malaria did not develop in any of the 6 test subjects when given 2 tablets in a single dose, weekly, for 8 weeks prior to and for 10 weeks after they had been bitten by infected mosquitoes. Radical cure in all of the 9 test subjects was obtained when the same dose was given weekly or bi-weekly for 10 doses following treatment of the acute attack with amodiaquin. The latter drug removed gametocytes from the circulating blood within 36 hours. It would thus appear that this preparation (Camoprim®) might well be used in malaria eradication programs. It has also been demonstrated that it is effective in obtaining a radical cure in individual cases.

ACKNOWLEDGMENT

The authors gratefully acknowledge the participation of the inmates of the Florida State Prison, Raiford, Florida, who freely volunteered and served as subjects for this trial. The co-

operation of the Governor of Florida and his Cabinet, the Superintendent of the State Prison, as well as the medical and auxiliary staff of the hospital were essential to the successful conclusion of the investigations and their contribution is greatly appreciated.

Grateful acknowledgment is also made to Dr. Martin D. Young, Laboratory of Parasite Chemotherapy, National Institute of Allergy and Infectious Diseases for supplying infective material (sporozoites) and to Doctors A. S. Alving and Robert Kellermeyer, Army Medical Research Center, Joliet, Illinois, for providing non-infected mosquitoes and infected blood.

The authors are also indebted to Dr. G. R. Coatney for his technical advice concerning the design of these trials and also to Mr. J. E. Meisenhelder for his generous laboratory technical assistance.

The friendly advice of Dr. P. E. Thompson, Laboratory Director of Parasitology, Department of Pharmacology, Research Division, Parke, Davis & Company, in designing and reporting this work is gratefully acknowledged.

REFERENCES

1. Edgcomb, J. H., Arnold, J., Young, Jr., E. H., Alving, A. S., Eichelberger, L., Jeffrey, G. M., Eyles, D. and Young, M. D., 1950. Primaquine, SN-13272, a new curative agent in *vivax* malaria, a preliminary report, *J. Nat. Malaria Soc.*, **9**: 285–292.
2. Alving, A. S., Hankey, D. D., Coatney, G. R., Jones, Jr., R., Coker, W. G., Garrison, P. L. and Donovan, W. N., 1953. Korean *vivax* malaria. II. Curative treatment with pamoquine and primaquine, *Am. J. Trop. Med. & Hyg.*, **2**: 970–976.
3. Coatney, G. R., Alving, A. S., Jones, Jr., R., Hankey, D. D., Robinson, D. H., Garrison, P. L., Coker, W. G., Donovan, W. N., Di Lorenzo, A., Marx, R. L., and Simmones, I. H., 1953. Korean *vivax* malaria. V. Cure of the infection by primaquine administered during long-term latency, *Am. J. Trop. Med. & Hyg.*, **6**: 985–988.
4. Arnold, J., Alving, A. S., Hockwald, R. S., Clayman, C. B., Dern, R. J., Beutler, E., Flanagan, C. L., and Jeffery, G. M., 1955. The antimalarial action of primaquine against the blood and tissue stages of *falciparum* malaria (Panama P-F-6 Strain). *J. Lab. & Clin. Med.* **46**: 391–397.
5. Arnold, J., Alving, A. S., Hockwald, R. S., Clayman, D. B., Dern, R. J., Beutler, E. and Jeffery, G. M., 1954. The effect of continuous and intermittent primaquine therapy on the relapse rate of the Chesson strain *vivax* malaria. *J. Lab. & Clin. Med.*, **44**: 429.
6. Alving, A. S. and Brucker, K., 1958. Observations on primaquine in the cure of *vivax* malaria. *Proc. VI Internat. Congr. Trop. Med. & Malaria* (in press).
7. Jeffery, G. M., Young, M. D. and Eyles, D. E., 1956. The treatment of *Plasmodium falciparum* infection with chloroquine, with a note on infectivity to mosquitoes of primaquine and pyrimethamine treated cases. *Am. J. Hyg.*, **64**: 1–11.
8. Penido, H. M., De Sousa, E. F. and Bezerra, Jr., F. P. G., 1948. Estudo sôbre a ação de novas drogas antimaláricas no Vale do Rio Doce. *Rev. Serviço Especial Saude Pub.*, **2**: 23–36.
9. Mein, R. M. and Rosado, P. N. S., 1948. Experiência com novos medicamentos contra a malária no programa de Amazônia. *Rev. Serviço Especial Saude Pub.* **1**: 1059–1091.
10. Halawani, A., Bax, I., and Morcos, F., 1947. A preliminary report on the anti-malarial Camoqui. *J. Roy. Egyptian M. A.*, **30**: 99–103.
11. Halawani, A., Bax, I., and Morcos, F., 1948. Field trials with new antimalarial drugs in Egypt. *Ann. Trop. Med. & Parasitol.*, **42**: 304–311.
12. Simeons, A. T. W., and Chhatre, K. D., 1947. Preliminary report on a new synthetic antimalarial. *Indian M. Gaz.*, **82**: 255–257.
13. Coatney, G. R., Cooper, W. C., White, W. C., Lints, H. A., Culwell, W. B. and Eyles, Don E., 1950. Studies in Human Malaria XXIV. Protective and therapeutic trials of SN 10,751 (Camoquin) against the Chesson strain of *Plasmodium vivax*. *J. Nat. Malaria Soc.*, **9**: 67–74.
14. Walker, A. J., 1955. Potentialities of monthly doses of Camoquin and a gametocidal drug in malaria control. *Tr. Roy. Soc. Trop. Med. & Hyg.*, **49**: 351–355.
15. Mitra, P. and Chaudhuri, R. N., 1955. Report on combined Camoquin and primaquine in the prophylaxis of malaria. Bull. Calcutta School Trop. Med., **3**: 46–47.
16. Coatney, G. R., 1955. The present status of chloroquine, pyrimethamine (Daraprim) and primaquine. *Industry & Trop. Health*, **2**: 116–122.

HUMATIN IN INTESTINAL AMEBIASIS

S. BELL AND A. W. WOODRUFF

London School of Hygiene and Tropical Medicine and Hospital for Tropical Diseases, London

As part of a program of trials of amebicidal drugs under standardized conditions, Humatin (Paromycin), prepared by Parke, Davis and Company, has been used in the treatment of 20 patients who had intestinal amebiasis and the results are here reported.

Humatin is an antibiotic consisting of a white, amorphous, water-soluble, stable substance, obtained from filtrates of submerged, aerated cultures of *Streptomyces*. It was made available for oral administration in the form of a partial sulphate.

In toxicity trials carried out by the makers it was administered to animals orally and parenterally and toxic effects were not observed after oral administration, for very little of the drug is absorbed. In each of two monkeys an oral dose was given, equivalent to 200 mg per kg of body weight, and the level of the drug in the blood was estimated after intervals of 2, 4, 6, and 24 hours. In the blood of one monkey, Humatin could not be detected, while in the second a concentration of 2.4 μg per 100 ml was found 2 hours after the dose had been given. Intravenous administration to rats and to monkeys resulted in mild but variable degrees of damage to the kidneys. In monkeys such changes were found following daily doses, given by intravenous injection, of 25 mg per kg of body weight for 17 days, followed by daily increments of the dose by 5 mg per kg of body weight until, on the last day of the experiment, the dose was 45 mg per kg of body weight. Comparison of Humatin with neomycin when given to rats by subcutaneous injection, showed that Humatin was the less nephrotoxic.

Humatin has been found to destroy *Entamoeba histolytica* in infected dogs, when used in doses as small as 3.13 mg per kg of body weight daily for 10 days. It is not yet known whether this effect is the result of a directly amebicidal action or to destruction of the bacteria with which the amebae grow in symbiosis. The drug, however, is at least fifty times more active against amebae than is neomycin, yet the antibacterial activity of the two is similar. Nevertheless, no action on amebae was found in concentrations of Humatin which failed to affect the bacteria in a mixed culture of amebae and bacteria.

In various human infections, daily doses as high as 75 mg per kg of body weight have been found by the makers to be free from toxic effects.

METHODS

Patients were admitted to the Hospital for Tropical Diseases, London. Diagnosis of amebic infection of the intestine was made by fecal examinations, both directly, and by concentration using the ether centrifugation method of Ridley and Hawgood.[1] Size of cysts of *E. histolytica* was measured with an eyepiece micrometer. The drug was given orally, in capsules, and in divided doses totaling 30 mg per kg of body weight daily. During treatment, stools were examined daily by the concentration technique in 14 cases, to discover when the parasites disappeared. Blood examinations, including estimation of the hemoglobin level, the erythrocyte, total leukocyte and differential counts and urine examination for the presence of protein and casts, were made before and after treatment. Following treatment the patients were instructed to attend the Out-patient Department at monthly intervals for assessment and for examination of a fecal specimen by the concentration technique. The majority had acquired their infection in India or tropical Africa, and either had alimentary symptoms when first seen, or had a history of such symptoms in the recent past.

PARASITOLOGICAL RESULTS

Twelve men, one boy, and seven women were found to be passing cysts of *E. histolytica* in their stools before treatment.

Time required for feces to be cleared of parasites. In the 14 patients whose feces were examined daily during treatment, cysts of *E. histolytica* disappeared after an interval of which the mean was 3.7 days after administration of the first dose.

Results of follow-up examinations. The results of the parasitological examinations are shown in Table 1. One patient relapsed a month after completing treatment. One patient did not attend for

follow-up examination. In one patient, in whose lower bowel there had been hemorrhagic lesions at the time of diagnosis, the lesions were still present 1 week after completing the course of Humatin, and although no amebae were found in the feces or in material scraped from the lesions, the absence of healing suggested that infection deep in the bowel mucosa had not been eradicated. A course of treatment with emetine bismuth iodide was followed by complete resolution of the lesions. The remaining 17 patients have each been examined on an average of 2.8 occasions ranging from 1 to more than 12 months after completion of treatment, and none has relapsed.

SIDE EFFECTS OF TREATMENT

Alimentary. The only adverse side effect noted was diarrhea commencing during treatment in six of the 20 patients. In three, this was mild, with up to 4 stools a day from the 3rd or 4th day of treatment. In the other three patients there was more severe diarrhea, with up to 10 stools a day. All these patients reported that the diarrhea ceased a few days after they completed the course of treatment.

Blood. It was found that Humatin exerted no particular effect on the blood. *Hemoglobin level*—The mean hemoglobin level of 19 patients before treatment was 95.8% (14.2g per 100 ml) and after treatment 97.5% (14.4g per 100 ml). *Erythrocyte count*—The mean erythrocyte count of 11 patients before treatment was 4.1 million per cu.mm and after treatment 4.6 million per cu.mm. *Leukocyte count*—The mean leukocyte count of 18 patients before treatment was 4,850 per cu.mm and after treatment 6,333 per cu.mm. *Differential leukocyte count*—Among 18 patients the mean percentages for each type of leukocyte before and after treatment respectively were: Neutrophils, 58% and 55.5%; lymphocytes, 34.3% and 35.4%; monocytes, 3.8% and 3.2%; eosinophils, 3.7% and 5.6%; and basophils, 1.0% and 1.0%. One patient was receiving treatment for a filarial infection simultaneously with anti-amebic treatment, and this accounts for the rise in the proportion of eosinophils shown here.

Urine. Examination of the urine of 17 patients before and after treatment showed no protein or casts. One man developed slight hematuria after having Humatin for 6 days. The course was suspended and a full urological investigation carried out. A minor lesion of the posterior urethra was found, which, in the opinion of the surgeon, would account for the bleeding. The course was completed after a 6-day interval, without untoward effects.

CONCLUSIONS

In the doses used, Humatin was shown to be an effective amebicide in chronic infections in which cysts were being passed in the feces, but ulcers in the large bowel may be slow to heal and the diarrhea which the drug sometimes provokes is troublesome. The dosage used in this trial appears to be near the minimum justifiable, for the relapse rate following it (5.6%) is higher than that obtainable after treatment with standard courses of emetine bismuth iodide.[2]

SUMMARY

1. Twenty patients who had *Entamoeba histolytica* cysts in their feces were treated for 10 days

TABLE 1

Number of patients treated, race of Entamoeba histolytica *with which patients were infected, intervals after treatment when examined parasitologically and results of such examinations*

Type of *E. histolytica* cysts present	Number of patients treated	Not followed	Number of patients followed up and results of fecal examinations									
			1 month		2 months		3 months		6 months		12 months	
			Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive
Large race*	13	1	4	1	5	0	6	0	5	0	4	0
Small race	3	0	1	0	1	0	1	0	2	0	1	0
Both races	4	0	1	0	4	0	2	0	2	0	1	0
Totals	20	1	6	1	10	0	9	0	9	0	6	0

* Cyst diameter 10μ or more; small race, under 10μ in cyst diameter.

with 30 mg Humatin per kg of body weight daily.

2. Among 14 of the patients, whose stools were examined daily during treatment, the mean time required for them to be cleared of cysts of *E. histolytica* was 3.7 days.

3. Nineteen patients have been followed up for periods ranging from 1 month to over 1 year after treatment and one has relapsed.

4. Six patients suffered from diarrhea which was apparently a side effect of the drug. The diarrhea ceased soon after completion of treatment.

5. No evidence of side effects of the drug on the blood or urine was obtained in these patients.

ACKNOWLEDGMENT

We wish to thank Dr. D. S. Ridley, Pathologist to the Hospital for Tropical Diseases, and his staff, for much laboratory help and Dr. H. W. Pearson, of Parke, Davis & Company, for information and for the supplies of the drug.

REFERENCES

1. Ridley, D. S., and Hawgood, B. C., 1956. The value of formol-ether concentration of faecal cysts and ova. *J. Clin. Path.*, **9**: 74–76.
2. Woodruff, A. W., Bell, S., and Schofield, F. D., 1956. The treatment of intestinal amoebiasis with emetine bismuth iodide, glaucarubin, dichloroacet-hydroxy-methyl-anilide, camoform and various antibiotics. *Tr. Roy. Soc. Trop. Med. & Hyg.*, **50**: 114–127.

SENSITIVITY OF LEBANESE AND SYRIANS TO TOXOPLASMIN AND TO A SIMILAR ANTIGEN PREPARED FROM *LEISHMANIA TROPICA**

KAMAL ABOU DAOUD AND CALVIN W. SCHWABE

Department of Tropical Health, Schools of Public Health and Medicine, American University of Beirut, Beirut, Lebanon

Although toxoplasma were reported in dogs in Baghdad,[1] to our knowledge toxoplasmosis has not been diagnosed in Lebanon or Syria, and statistics are not presently available for the two countries on the frequency of positive reactors to either the standard serological tests or to the toxoplasmin skin test. Physicians at the American University Hospital are aware, however, of clinical syndromes suggestive of the disease. The single, strongly presumptive case known to us occurred in a 2-month-old male Moslem from Damascus, admitted to the American University Hospital in June, 1957, with irregular fever and decreased activity since birth. On examination, numerous brain calcifications, microphthalmia, chorioretinitis, nystagmus, hepatosplenomegaly, spasticity of the upper extremities and hyperactive reflexes were found. Complement fixation tests and Sabin-Feldman dye tests performed at the University of Zurich, Switzerland, were positive at titres of 1:5 and 1:4000 respectively on the infant and at 1:40 and 1:16,000 respectively on the mother.†

Through the courtesy of Dr. P. L. Kurtz of Eli Lilly and Company, a supply of toxoplasmin antigen and control was recently made available for a small survey among nationals of Lebanon and of the Syrian region of the United Arab Republic. One hundred twenty-nine persons were tested in all, 78 of whom were chosen at random and 51 of whom were selected because of known previous infection with *Leishmania tropica* (as evidenced by the presence of one or more typical scars on exposed portions of the body). In so doing we hoped to obtain as satisfactory an estimate of toxoplasmin sensitivity as a small sample would permit and at the same time to learn whether past infection with the prevalent intracellular protozoan, *Leishmania tropica*, could be expected to result in false positive or other misleading reactions to *Toxoplasma gondii* antigen.

* Portion of a study on the epidemiology of zoonotic diseases in the Middle East supported by a grant from Pfizer International. Mrs. Euphronia Meymerian assisted in the preparation of the bibliography; Mr. John Himo and Mr. Salim Lutfallah provided technical assistance.

† For permission to cite this case we are grateful to Drs. Hans Zellweger and B. Andonian, Department of Pediatrics, American University Hospital.

MATERIALS AND METHODS

Eli Lilly's toxoplasmin antigen and control were administered intracutaneously as directed. Similarly, each individual received a toxoplasmin-like antigen prepared from *Leishmania tropica* ("leishmanin") as well as its appropriate control. Both tests were read after 36–48 hours. Considered positive were those which gave 1 cm or more of erythema and induration with no reaction to the control.‡

The leishmanial antigen was prepared from organisms isolated on N.N.N. medium from local human cases of cutaneous leishmaniasis. Leptomonads from rich N.N.N. subcultures were aseptically collected in small amounts of physiological saline and concentrated by centrifugation. Distilled water was added in an amount equal to 10 times the wet weight of leptomonads and the latter were lysed by freezing and thawing 10 times. Final dilution to 1:1000 was made in 0.87% saline containing 0.25% phenol. Sterility tests were done in fluid thioglycollate medium at 37°C, on N.N.N. medium at 30°C and by direct microscopic examinations. A control was prepared from saline washings of uninoculated tubes of N.N.N. medium which had been similarly handled.

RESULTS

Of the 129 persons tested, 73 or 57% were positive to toxoplasmin. All persons were in Lebanon at the time of the test. Of the 72 whose homes were in Syria or who had resided there for extended periods, 47 or 65% were toxoplasmin positive. Of 57 Lebanese, 26 or 46% gave positive

‡ One but less than 2 cm was considered +, 2 but less than 3 cm ++, 3 but less than 4 cm +++ and 4 cm or more ++++.

TABLE 1

Toxoplasmin and leishmanin sensitivity in individuals without leishmanial scars

		Age							Sex		Nationality		Religion			Reaction
		0-9	10-19	20-29	30-39	40-49	50-59	60-69	Males	Females	Lebanese	Syrians	Christians	Moslems	Druses	
Number of persons tested	78	2	10	50	9	2	2	3	57	21	56	22	57	15	6	
Toxoplasmin positive individuals	40	0	4	27	4	1	1	3	28	12	25	15	28	10	2	19 + 17 ++ 3 +++ 1 ++++
Leishmanin positive individuals	9	0	4	2	2	0	1	0	7	2	4	5	7	1	1	5 + 3 ++ 1 ++++
Individuals both toxoplasmin and leishmanin positive	7	0	4	1	1	0	1	0	5	2	3	4	5	1	1	

TABLE 2

Toxoplasmin and leishmanin sensitivity in individuals with leishmanial scars

		Age						Sex		Nationality		Religion			Reaction
		0-9	10-19	20-29	30-39	40-49	50-59	Males	Females	Lebanese	Syrians	Christians	Moslems	Druses	
Number of persons tested	51	0	6	30	7	7	1	29	22	1	50	43	8	0	
Toxoplasmin positive individuals	33	0	5	19	5	4	0	20	13	1	32	27	6	0	25 + 7 ++ 1 ++++
Leishmanin positive individuals	46	0	6	27	7	5	1	26	20	1	45	39	7	0	12 + 31 ++ 2 +++ 1 ++++
Individuals both toxoplasmin and leishmanin positive	30	0	3	18	5	4	0	17	13	1	29	25	5	0	

reactions. More detailed treatments of data appear in Table 1 and 2.

The two groups considered were in certain respects not comparable; those in Table 1 were more nearly representative of the general population, while the Table 2 group included a disproportionately high number of Syrian Christians, non-Arabs (mostly Armenians) and residents of Aleppo and vicinity. (Aleppo is, of course, a well-known endemic focus of cutaneous leishmaniasis.) Only a single individual included in Table 2 had never been outside of Lebanon.

Forty of the 51 persons with leishmanial scars were able to give their age at the time of infection. The range was 3–15 years; the mean age at infection was 7.2 years.

With respect to toxoplasmin sensitivity there seemed to be no difference attributable to sex. The sensitivity rate among the Syrians tested was higher than that among the Lebanese; this reflects also in the apparent difference in toxoplasmin sensitivity between the group with and the group without leishmanial scars. There would seem to be no evidence of cross-sensitization between the 2 infections. (The single curious statistic is that while only 51% of all the persons included in Table 1 were toxoplasmin-positive, among the 9 leishmanin-positive individuals of this group, 7 or 78% were positive to toxoplasmin. The number of individuals is unquestionably too small for any conclusion.)

Sixteen of 23 or 70% of the Moslems tested were toxoplasmin-positive as compared to 55 of 100 or 55% of the Christians. This statistic, while based on a very small number of Moslems, is not biased by age or by country of residence (11 of the Moslems were Syrians and 12 Lebanese). Of interest were the 9 leishmanin-positive individuals without leishmanial scars. Of these, one had lived in an endemic area in Turkey or in Aleppo for 40 years, a second in Aleppo for 23 years, a third for 20 years, a fourth for a shorter period of time and a fifth had a doubtful scar on the wrist. One of the four others gave a ++++ reaction to leishmanin. She had lived in endemic areas of Syria and Iraq for 24 years and had been inoculated repeatedly, as a volunteer, with living leptomonads of *Leishmania tropica* without reaction (during a study by Berberian[2]).

Of interest, also, were the 5 individuals, among the 51 with leishmanial scars, who did not give a positive reaction to leishmanin. One of these, an Armenian Christian laborer, 21 years of age, is an unusual case.* This young man lived in Aleppo until the age of 6, and since that time in Lebanon. He contracted cutaneous leishmaniasis at 6 years and has had extensive and continuously active lesions of the face and ears since then. Despite intensive therapy, *Leishmania tropica* may still be isolated from his lesions.

DISCUSSION

Very little is known of toxoplasmosis in this area although recent experiences with the disease in Europe and the United States have made a number of our clinicians *Toxoplama*-conscious. To our knowledge *Toxoplasma gondii* has yet to be isolated from any host species. Nevertheless the high rate of toxoplasmin sensitivity in the local population suggested by this survey is not inconsistent with results obtained by others elsewhere.

One point of epidemiologic interest concerns pork, which is not commonly eaten by most Christians, and never eaten by most Moslems, in this part of the world. Although, as suggested a few years ago by several authors (see Jacobs[3]), swine and their products may be significant factors in the epidemiology of toxoplasmosis elsewhere, they are unlikely to be so here.

Cutaneous leishmaniasis, on the other hand, has been recognized in the fertile crescent sufficiently long to be well-known as both Aleppo button and Baghdad boil. An epidemiologic study of the leishmaniases in Lebanon, Syria, Palestine and Iraq was made by Adler and Theodor in 1929,[4] and more recent epidemiologic data are included in Simmons *et al.*[5] Human cutaneous leishmaniasis occurs in an important focus at Aleppo and in several lesser foci in both Syria and Lebanon. Apparently autochthonous cases of human visceral leishmaniasis occur sporadically. Despite the early negative reports,[4] canine visceral infection is present. In a recent survey of some 150 street dogs from Beirut and other Lebanese towns, Dr. Emile Rizk (then a member of this Department) found, by spleen, bone marrow and lymph node smears, 3 infections with *Leishmania* sp. Two additional visceral infections, in dogs from a Lebanese village, were brought to our attention 2 years ago by Dr. Joseph Asmar, Associate Professor of Veterinary Science in this University. (Canine visceral infection in Iraq has been reported recently by Sheriff).[6]

Infection with *Leishmania* has not been seen here in other host species, however, although attempts have been made during the last 3 years to isolate the organism on N.N.N. medium from the spleens of 285 local mammals (representing 16 species) which have been collected by and autopsied in this Department (Schwabe and Schinazi, unpublished data).

The use of leishmanial skin test antigens has been reported previously,[7-12] more recently as aids in the diagnosis of active mucocutaneous leishmaniasis. In this connection, Biagi[13] has mentioned that there is apparently no cross-sensitization between this latter disease and toxoplasmosis.

* This patient was brought to our attention by Dr. Hrant Chaglassian, Professor of Dermatology, American University of Beirut.

SUMMARY

1. Fifty-seven per cent of 129 Lebanese and Syrians tested with toxoplasmin gave positive reactions. Sixty-five per cent of the Syrians and 46 per cent of the Lebanese were positive. Seventy per cent of Moslems tested were toxoplasmin-positive as compared to 55 per cent of Christians.

2. Of 51 individuals with leishmanial scars, 46 gave positive skin reactions to a toxoplasmin-like antigen prepared from *Leishmania tropica*. Nine individuals without leishmanial scars were also positive to the leishmanial antigen.

3. There appeared to be no cross-sensitization to skin test antigens prepared from *Toxoplasma gondii* and *Leishmania tropica*.

4. Presently available information concerning the epidemiology of toxoplasmosis and leishmaniases in Lebanon and Syria is briefly discussed with particular regard to recent observations.

REFERENCES

1. MacHattie, C., 1938. Notes on two cases of naturally occurring toxoplasmosis of the dog in Baghdad. *Trans. Roy. Soc. Trop. Med. & Hyg.*, **32**: 273–276.
2. Berberian, D. A., 1939. Vaccination and immunity against oriental sore. *Tr. Roy. Soc. Trop. Med. & Hyg.*, **33**: 87–94.
3. Jacobs, L., 1957. The interrelation of toxoplasmosis in swine, cattle, dogs and man. *Pub. Health Rep.*, **72**: 872–882.
4. Adler, S., and Theodor, O., 1929. The distribution of sandflies and leishmaniasis in Palestine, Syria and Mesopotamia. *Ann. Trop. Med. & Parasitol.*, **23**: 269–306.
5. Simmons, J. S., Whayne, T. F., Anderson, G. W., Horack, H. M., and Thomas, R. A., 1954. *Global Epidemiology*. Vol. 3, The Near and Middle East. Philadelphia, London and Montreal, J. B. Lippincott Company.
6. Sheriff, D., 1957. Note. Canine visceral leishmaniasis in foxhounds near Baghdad. *Tr. Roy. Soc. Trop. Med. & Hyg.*, **51**: 467.
7. Wagener, E. H., 1923. A skin reaction to extracts of *Leishmania tropica* and *Leishmania infantum*. *Univ. Calif. Pub. Zool.*, **20**: 477–488 (Abstract: *Trop. Dis. Bull.*, **22**: 202).
8. Montenegro, J., 1926. Cutaneous reaction in leishmaniasis. *Arch. Dermat. & Syph.*, **13**: 187–194.
9. Dostrovsky, A., 1935. The diagnostic value of leishmania vaccine. *Ann. Trop. Med. & Parasitol.*, **29**: 123–128.
10. Dostrovsky, A., and Sagher, F., 1946. The intracutaneous test in leishmaniasis. *Ann. Trop. Med. & Parasitol.*, **40**: 265–269.
11. Echandi, C. A., 1953. Estudios sobre la sensibilidad cutánia en la leishmaniosis tegmentaria en Costa Rica. *Riv. Biol. Trop. San José*, **1**: 173–195 (Abstract: *Trop. Dis. Bull.*, **51**: 784).
12. Floch, H., and Casile, M., 1955. Intradermoréaction de Monténégro a la leishmanine. *Bull. Soc. path. exot.*, **48**: 636–642.
13. Biagi, F. F., 1953. Intraderma reacciones con leishmanina en Escárcega, Camp. (Méx.). *Medicina*, Mexico, **33**: 255–260 (Abstract: *Trop. Dis. Bull.*, **50**: 1031).

SOME QUANTITATIVE DATA ON THE GROWTH OF *ENTAMOEBA HISTOLYTICA* FROM SINGLE-CELL ISOLATIONS IN MICROCULTURES.*

CHARLES W. REES, IRIS D. KEY AND JAMES G. SHAFFER

From the Department of Microbiology and Public Health, The Chicago Medical School, Chicago 12, Illinois

The investigations reported in this paper were conducted with the use of a micromanipulator designed by Rees for the specific purpose of isolating certain protozoa from bacteria. It has been used for microisolation of *Trichomonas foetus* and cysts of *Entamoeba histolytica*.[1–3] Reardon and Bartgis[4] isolated cysts of a small-race of *E. histolytica* from a stool specimen and established this strain, designated as NIH 110, with organism *t* in whole egg medium. Rees, Reardon and Bartgis[5] obtained bacteria-free excystation of a strain of large race by isolating cysts in an anaerobic environment. Attempts to isolate trophozoites of the ameba have presented considerable difficulties, even from monoxenic cultures as defined by Dougherty.[6] It has not been possible to study the metabolism of amebae from monoxenic cultures in microtubes because of gas production, usually carbon dioxide and hydrogen. Von Brand *et al.*[7] showed that these fermentations of reducing substances were attributable to the bacterium.

Shaffer and associates[8–12] have produced cultures, designated as "S-F," with a bacterium as yet unidentified, which probably do not conform to Dougherty's definition of "monoxenic" since measurable reactions such as production of acid, gas, or changes in pH, do not occur during the period of incubation.

In addition, the work of Shaffer includes production of cultures of the ameba without bacteria in medium containing embryonic material from the chick.[13, 14] As an outgrowth of the development of the S-F medium, cultures of the ameba with *Trypanosoma cruzi* were produced by Phillips.[15] Bacteria-free cultures of *E. histolytica-T. cruzi* produced from microisolated cysts were used in investigations on amebiasis in germ-free animals at Notre Dame University.[16] Phillips and Rees[17] studied *E. histolytica* with this trypanosome from single-cell isolations in microtubes. Baernstein *et al.*[18] conducted extensive investigations on the growth of single-cell isolations of the ameba in the chick-embryo medium with a number of qualitative and quantitative modifications of the ingredients. All ingredients prescribed by Shaffer *et al.* were found essential. Some components including serum could withstand heating slightly beyond 50°C and products lost through dialysis could be restored in part by a formula of vitamins, but the embryonic materials were thermolabile.

The present investigation was concerned with the behavior and growth of the ameba from single-cell isolations into the Shaffer-Frye (S-F) medium which can be prepared and maintained under uniform conditions and with transfer of the progeny to medium in test tubes, rather than with results of experimental alterations of medium. One objective was to furnish a basis for comparison with results of experiments on growth factors, pathogenicity, and other attributes of this pathogenic ameba. Another objective was to establish "pure lines" by transfer of microcultures to test tubes.

MATERIALS AND METHODS

The S-F medium is prepared as follows: An as yet unidentified Gram-negative anaerobic bacillus is incubated for 24 hours at 37°C in fluid thioglycollate medium (Baltimore Biological Laboratories No. 136C) containing 1.0% glucose and 1.0% rice starch. This culture is then centrifuged horizontally (International PR 2) at 2,000 rpm for 45 minutes. The supernatant fluid, which is known to contain 30 to 70 million viable bacterial cells per ml, is collected and used as the base for the medium. Of this fluid, 2.5 ml are added to culture tubes containing 0.1 ml normal horse serum and 0.7 ml saline (0.85% NaCl) which contains 1,000 units of penicillin G potassium. The medium is then inoculated with appropriate material containing *E. histolytica* and layered

* This investigation was supported by a research grant from the Institute of Allergy and Infectious Diseases (E-499C5) of the National Institutes of Health, Public Health Service, U. S. Department of Health, Education and Welfare.

with petrolatum to aid in the maintenance of anaerobic conditions.

In some of the experiments to be described medium was used 24 to 72 hours after it was made up. It is possible to do this by sealing the tube with petrolatum and keeping it at 37°C until needed. This tube is conveniently referred to as a "blank" since it does not contain amebae. The strain of *E. histolytica* used in the present investigation, designated as K-9, was brought from Korea in 1951 by Dr. W. W. Frye and obtained by us from Dr. M. M. Brooke of the Communicable Disease Center of the U. S. Public Health Service.

Problems connected with microisolation of cysts differ from those involved in isolation of trophozoites from the S-F medium, in that for various reasons, separation from the bacillus is not required. It is, however, necessary to use special precautions to prevent irreversible damage to the amebae during passage into and out of the micropipette. The ameba is picked from the "mother culture," transferred to experimental medium, washed to eliminate carry-over substances, and finally transferred to a microtube of the medium. The microtube is sealed with petrolatum and is examined for amebae by direct observation while immersed in water to reduce disturbing reflections. The microtubes and methods illustrated in Figure 1 (a), as used by Baernstein *et al.*[18] were not used by us because amebae and medium were exposed to oxygenation during considerable periods of time. Such exposure was reduced to a minimum by use of the microtube shown in Figure 1 (b), which was filled with medium, "threaded" on to the micropipette and inoculated with an ameba. Other methods designed to avoid insertion of the pipette into the microtube are illustrated in Figure 2. The microtube consisted of the cut-off end of the micropipette in one case (a), and a piece of tubing sealed with Zip wax in the other (b). Other operations, as well as that of "pumping" a microculture into a test tube (Figure 3), are self-explanatory. The use of Luer syringes with petrolatum-greased plungers to hold medium and inoculum until the time of the operations of microisolation has further reduced the time of exposure to air.

A set of 5 microcultures was usually prepared for each experiment. These were examined shortly after completion to make sure that each

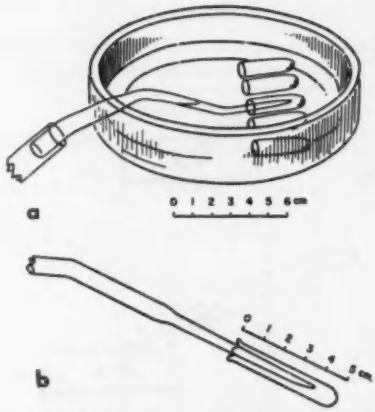


FIG. 1. Methods of preparing microcultures from single amebae injected into microtubes from the micropipette. *a.* Microtubes filled with experimental medium and immersed in Hank's solution were inoculated with one ameba that had previously been taken into the micropipette. *b.* Microtube was threaded in air onto the micropipette containing an ameba. Injection of the ameba was done in air or subsequent to immersion of the microtube in liquid.

contained a motile trophozoite. Any microculture in which no ameba could be seen or in which the ameba it contained appeared to have been damaged, was immediately replaced.

Preliminary experiments showed that, in 1 or more of each set of 5 microcultures, there was rapid division of the isolated amebae (more than 17 generations, 128 progeny, during 72 hours of incubation) and that pure lines were produced with regularity, in test tubes from our method of transfer. The results of an experiment presented in Table 1 show that there was considerable variation in numbers of the progeny from different microtubes at all 3 periods of incubation and a lack of correlation among the numbers at early and later periods. Such variation is far beyond what might be expected from an organism so well defined as *E. histolytica* when grown under uniform conditions. Further experiments were therefore conducted in attempts to ascertain the nature of the factors responsible for the excessive variation. However, despite all attempts to eliminate variable factors the results did not show appreciable improvement over those of Table 1.

The data from a considerable number of sets of microcultures are summarized in Table 2, and illustrate the extent of the variation over a series

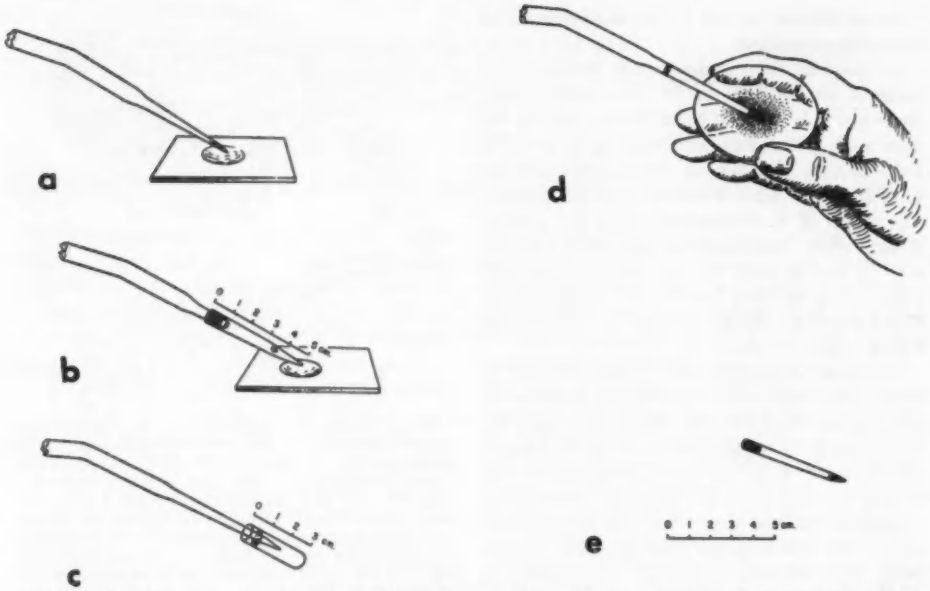


FIG. 2. Methods of preparing microcultures from single amebae taken directly into tubing which was later converted into microtubes. *a*. Use of the tip of the micropipette as a microtube. A single ameba in a drop of medium was drawn into the pipette which was then cut off and sealed. *b*. A microtube was sealed onto the cut-off end of the micropipette and a single ameba withdrawn into the microtube from a drop of medium on a sterile slide. *c*. Experimental medium was drawn into the microtube from a sterile container. *d*. The tip of the microculture was sealed with warmed petrolatum. *e*. The microculture was completed by sealing the other end with petrolatum. This was the end result of procedures illustrated in *a*, *b*, *c*, and *d*.

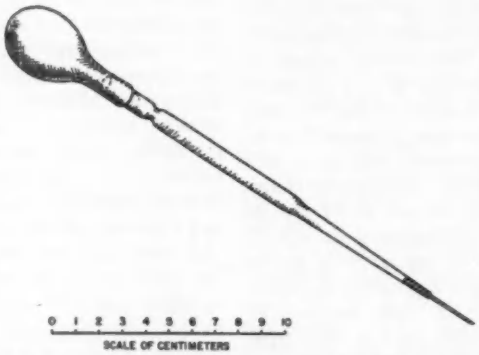


FIG. 3. Method of connecting a microculture to a Pasteur pipette for transfer of the amebae to a test tube to establish a microculture.

of experiments. The results reveal an average 5-fold increase in the progeny between 24 and 72 hours of incubation for microtubes listed in the upper portion compared with an almost 20-fold increase for those of the lower portion. Low yields at 72 hours cannot be explained on the basis of the numbers of progeny at 24 hours which were almost as high in tubes with low final yields as in those showing remarkably high rates of reproduction. These differences cannot

TABLE 1

Results of an experiment on growth of amebae from single-cell isolations in microtubes and transfer to medium in test tubes

Microtube number	Progeny at indicated periods of incubation			Results of transfer to test tubes at indicated hr.
	24 hrs	48 hrs	72 hrs	
1	8	25	30	96 hrs Pos. at 24 hrs
2	5	24	82	96 hrs Pos. at 48 hrs
3	3	30	109	96 hrs Pos. at 48 hrs
4	12	60	105	96 hrs Pos. at 48 hrs
5	1	18	18	96 hrs Pos. at 48 hrs
6	4	52	130	72 hrs Pos. at 24 hrs
7	11	46	137	72 hrs Pos. at 24 hrs
8	1	45	124	72 hrs Pos. at 24 hrs
9	3	8	60	72 hrs Pos. at 48 hrs
10	5	28	88	72 hrs Pos. at 24 hrs
11	3	28	12	72 hrs Neg.*
12	2	12	75	72 hrs Pos. at 24 hrs

* Not demonstrable by regularly used method of examination through walls of test tube but found on examination of sediment.

be explained by the use of different "blanks" because they occurred in all sets of 5 microtubes and each set was filled from one blank. In attempting to explain the wide variation seen in these microcultures, a number of possibilities have been considered. (1) There may be an uneven distribution of particulate material to the various microtubes. Previous reports have indicated that the growth material in the S-F medium is particulate.[10, 11] Thus, one microtube may receive only enough to support propagation for 24 hours, but not enough for continued propagation. (2) There may be inherent differences (genetic) in the individual amebae. (3) There may be undetectable damage done to the trophozoite during micromanipulation. (4) One may not always select an ameba during phases of active multiplication. In studies on division of *Trypanosoma lewisi* Taliaferro[19] demonstrated that rapidly dividing trophozoites exhibited great variation in size whereas those failing to divide were of uniform length. A similar situation may exist with *E. histolytica*.

Information on the question to what extent the variations in yields of amebae from medium prepared under standardized conditions might affect the evaluation of the results from certain experimental changes of medium is summarized in Table 3. The medium was adjusted at different levels of pH. Changes either up or down the pH scale had marked effects on the yields with variations far wider than those obtained from the standardized medium. However, certain tubes at high levels of pH showed good yields. The results

TABLE 2

Progeny of single amebae, selected from experiments conducted over a period of several months

Microtube number*	Progeny of single amebae at indicated periods of incubation		
	24 hrs.	48 hrs.	72 hrs.
1	9	0	0
2	7	6	0
3	2	6	1
4	4	9	16
5	5	12	17
6	4	10	20
7	8	48	30
8	5	20	38
9	9	15	38
10	10	45	42
11	3	12	48
12	1	8	50
13	4	19	60
14	10	38	63
Averages.....	6	17	30
15	12	30	66
16	9	40	72
17	7	28	83
18	12	24	86
19	10	24	94
20	2	16	98
21	12	50	98
22	6	51	112
23	12	26	116
24	7	28	140
25	11	103	175
26	10	45	205
27	12	103	289
28	16	124	300
29	10	28	321
30	13	140	325
31	8	50	400
Averages.....	9	53	176

* The data of this table are arranged in the order of numbers of the progeny of single amebae at 72 hours of incubation, rather than dates at which the experiments were conducted.

TABLE 3

Progeny of single amebae in medium at indicated levels of pH

pH	Number of experiments	Total micro-cultures	Total progeny	Highest yield	Negative*	Average yield
5.4	3	15	3	—	12	—
6.0	2	10	470	225	4	47
6.4	control	5	525	250	0	105
7.0	4	23	625	169	7	27
7.4	4	28	168	140	10	6

* No amebae in the culture after 24 hours incubation.

indicate that under conditions of these experiments, maximum average yields occurred at a pH of 6.4. Shaffer,[10] using test tube cultures, obtained the maximum propagation at pH 6.0. Baernstein *et al.*[18] observed high yields at pH 7.9 using another medium. Such results would seem to suggest that the optimal pH for propagation of *E. histolytica* is determined by the medium or by other conditions, rather than by some exact requirement of the organisms.

DISCUSSION

The results reported above indicate that one can, with relative ease, establish pure lines of *E. histolytica* in culture by microisolation of individual trophozoites. It has, in these experiments, been found necessary to use the intermediate step involving propagation in the microtube. In the course of these experiments well over 50 attempts were made in which single trophozoites were introduced into test tubes containing the routine 3.5 ml of S-F medium without obtaining a single positive culture. On the other hand, the transfer from successful microcultures to macrocultures, as indicated in Table 1, was accomplished with relative ease. These observations were, of course, surprising, and as yet no reasonable explanation can be offered.

It was also surprising to observe the rather marked differences in the growth patterns obtained in the microcultures. As previously pointed out, there are several possible explanations for this variation which need further investigation.

It would seem that microisolation of single trophozoites offers a useful means by which genetic and metabolic properties of *E. histolytica* may be studied. With appropriate care one can wash such a trophozoite free of carry-over from the previous culture substrate so that valuable data may be obtained from a single operation. It must be remembered, however, that all washing procedures are subject to certain limitations, dictated in large measure by the fragility of the trophozoite. Thus, whether the washing is accomplished by this or by other means, the number of operations and the care with which they are carried on determine the freedom from carry-over and the success of the culture.

SUMMARY

A micromanipulator of original design was used for transfer of single amebae from the S-F medium in test tubes to microcultures. Quantitative data collected on the growth of the organisms under these circumstances revealed considerable variation in the total progeny after incubation of the microcultures even though all possible efforts were made to standardize the conditions. Attempts to transfer single amebae to test tube cultures failed to produce successful cultures, but it was found relatively easy to establish test tube cultures by transferring the microcultures to test tubes of S-F medium. Certain refinements were made in the microtechnique in the course of these experiments. It is suggested that microisolation of single trophozoites offers a good means of studying genetic and metabolic characteristics of *Entamoeba histolytica*.

REFERENCES

1. Rees, C. W., 1937. Obtaining bacteria-free pure lines of *Trichomonas foetus* by means of microisolation. *Am. J. Hyg.*, **26**: 283–291.
2. Rees, C. W., 1942. The construction of a micromanipulator for the isolation of protozoa. *Am. J. Trop. Med.*, **22**: 487–492.
3. Rees, C. W., 1955. *Problems in Amoebiasis*. Charles C Thomas, Springfield, Illinois.
4. Reardon, L. V. and Bartgis, I. L., 1949. The cultivation of the small-race *Endamoeba histolytica* wigh a single species of bacteria. *J. Parasitol.*, **35**: 218.
5. Rees, C. W., Reardon, L. V., and Bartgis, I. L., 1950. The excystation of *Entamoeba histolytica* without bacteria in microcultures. *Parasitology*, **40**: 338–352.
6. Dougherty, E. G., 1953. Problems on nomenclature for growth of organisms of one species with and without associated organisms of other species. *Parasitology*, **42**: 259–261.
7. von Brand, T., Rees, C. W., Jacobs, L., and Reardon, L. V., 1943. Studies on reducing substances and gas formation in cultures of *Endamoeba histolytica* and a single species of symbiotic bacteria. *Am. J. Trop. Med.*, **23**: 310–319.

8. SHAFFER, J. G., AND FRYE, W. W., 1948. Studies on the growth requirements of *Endamoeba histolytica*. I. Maintenance of a strain of *E. histolytica* through one hundred transplants in the absence of actively multiplying bacterial flora. *Am. J. Hyg.*, **47**: 214–221.

9. SHAFFER, J. G., 1952. Factors affecting the propagation of *Endamoeba histolytica* in vitro in the S-F medium and in tissue bearing substrate. *Ann. New York Acad. Sci.*, **56**: 1033–1047.

10. SHAFFER, J. G., 1952. Studies on the growth requirements of *Endamoeba histolytica*. V. Studies on the nature of some of the factors in the Shaffer-Frye medium that affect the propagation of *E. histolytica*. *Am. J. Hyg.*, **56**: 119–138.

11. SHAFFER, J. G., SCHULER, R. W., AND KEY, I., 1958. Studies on the growth requirements of *Entamoeba histolytica*. The ingestion of altered bacterial structures by *E. histolytica* in the Shaffer-Frye medium. *Am. J. Trop. Med. & Hyg.*, **7**: 302–308.

12. SHAFFER, J. G., RYDEN, F. W. AND FRYE, W. W., 1949. Studies on the growth requirements of *Endamoeba histolytica*. III. The growth and multiplication of two strains of *E. histolytica* in a transparent medium without addition of rice flour or other particulate matter and without demonstrable bacterial growth. *Am. J. Hyg.*, **49**: 127–138.

13. SHAFFER, J. G., AND SIENKIEWICZ, H. S., 1952. Propagation of a strain of *Endamoeba histolytica* in tissue bearing culture. *Science*, **116**: 306–308.

14. SHAFFER, J. G., SIENKIEWICZ, H. S. AND WASHINGTON, J. E., 1953. The propagation of *Endamoeba histolytica* in tissue-bearing culture without accompanying bacteria or other microorganisms. *Am. J. Hyg.*, **57**: 366–379.

15. PHILLIPS, B. P., 1950. Cultivation of *Endamoeba histolytica* with *Trypanosoma cruzi*. *Science*, **111**: 8–9.

16. PHILLIPS, B. P., WOLFE, P. A., REES, C. W., GORDON, H. A., WRIGHT, W. H. AND REYNIERS, J. A., 1955. Studies on the amebabacteria relationships in amoebiasis. Comparative results of intracecal inoculation of germ-free, monocontaminated, and conventional guinea pigs with *Entamoeba histolytica*. *Am. J. Trop. Med. & Hyg.*, **4**: 675–692.

17. PHILLIPS, B. P. AND REES, C. W., 1950. The Growth of *Endamoeba histolytica* with live and heat treated *Trypanosoma cruzi*. *Am. J. Trop. Med.*, **30**: 185–191.

18. BAERNSTEIN, H. D., REES, C. W., AND BARTGIS, I. L., 1957. The rate of reproduction of *Entamoeba histolytica* in microcultures from inocula of single trophozoites in cell-free medium prepared from embryos of the chick. *J. Parasitol.*, **43**: 143–152.

19. TALIAFERRO, W. H., 1929. *The Immunology of Parasitic Infections*. The Century Company, New York.

INTESTINAL PROTOZOA AND HELMINTHS IN SCHOOL CHILDREN OF DACCA, EAST PAKISTAN (EAST BENGAL)*

ROBERT E. KUNTZ

Naval Medical Research Unit No. 2, Taipei, Taiwan

The subcontinent of India has frequently experienced the ravages of communicable and other diseases on an epidemic scale. In the spring of 1958 the Provincial Government of East Pakistan, as a result of extensive occurrences of cholera and smallpox, made a plea for outside assistance. Medical supplies and technical help were offered by several nations. The Commanding Officer of U. S. Naval Medical Research Unit No. 2 provided technical assistance by offering the services of a group of investigators and technicians from the Unit at Taipei, Taiwan. The author, serving in the capacity of parasitologist, accompanied this group which was designated as the U. S. Naval Medical Mission to East Pakistan.

The present report concerns people in an area where there have been few surveys giving the incidence of parasites and other intestinal microfauna. Data and information were obtained from a study of stool specimens collected in the vicinity of Dacca, the capital. East Pakistan possesses peoples with varied cultures and backgrounds and the recent partitioning of India and the Pakistans has resulted in a continual two-way migration of refugees. Dacca, with a population of approximately one-third million, is located on the Burhi Ganga River in the vast alluvial plain formed by the silt desposits from the Ganges, Brahmaputra and Meghana Rivers. The climate is warm and humid and the average rainfall is reported as 80+ inches per year.

MATERIALS AND METHODS

Most of the fecal samples were obtained from school children 6 to 18 years of age with the majority in the 10 to 16 years age group. Although the work of the Naval Mission was concerned primarily with cholera which was prevalent in Dacca at the time, study materials for this report were obtained only from individuals apparently in a normal state of health. Stools were taken from three areas representing slightly different sociologic and ecologic habitats.

Mirpur Colony High School, a school with an enrollment of approximately 1100 students located a few miles north of Dacca in an area in which a good part of the population was engaged in farming, marketing and allied occupations. As a result of an influx of numerous refugees from India and other parts of Pakistan, a number of people were unemployed. Mirpur is located near a branch of the Burhi Ganga River and the population makes generous use of its waters. As in most villages in this part of the world, the people were crowded into limited living space thus encouraging the spread and propagation of certain diseases. The majority of the population was Muslim, the remainder Hindu. Questioning on the spot and macroscopic examination of stools revealed the local diet to consist mainly of rice and vegetables. Water for the village was available from tube wells but it is likely that water was taken from small ponds and rivers for household use.

Teijgaon Polytechnic High School, a specialized, semi-private institution with approximately 500 students is located in Dacca proper. The student body was primarily Muslim and most of the children came from homes in the urban community. The majority of parents were shopkeepers, merchants and civil servants. Since attendance at this school required tuition, it is reasonable to assume that the students came from a slightly different economic level than characteristic of most people of East Pakistan. This assumption was further supported by the general conditions and appearance of the school as well as of the students.

Demra Secondary School is a small inadequate school crowded with approximately 300 students. Stools were obtained from some parents and younger children as well as from the students. Since this series included several children and

* The opinions or assertions contained herein are the private ones of the author and are not to be construed as official or reflecting the views of the Department of the Navy.

Mail address: APO 63, San Francisco, California

parents, the ages ranged from 2 to 55 years. However, the majority fell within the 10 to 20 years age group. Most of the children were from families in which parents and older children were laborers at the nearby jute mills. The general laxness in sanitation, mode of living and a prolific fly population in the village suggested an ideal habitat for effective propagation and transmission of parasites. Villagers had direct access to the nearby Burhi Ganga River. As in other villages in this general area, tube wells were present but the nearby river apparently served as a source of water for general use.

Even with the aid of a well-trained and experienced interpreter only a very cursory background history (age, occupation of parents, etc.,) could be obtained. As in previous surveys, fecal samples were fixed by the "MIF-vial" method[1] which facilitates collection of study materials under adverse conditions and gave assurance of immediate and proper fixation of the included microfauna. The majority of specimens was removed from sputum cups and fixed within 2 to 4 hours after passage.

For collection in the field 15-ml capacity screw-cap vials were employed and 9.4 ml of MF (merthiolate-formalin) solution was added prior to departure from Taipei. Lugol's solution (0.6 ml) was added to MF a few seconds before addition of fecal sample to vial. When volume of stool permitted, three samples totaling about 1 ml were taken from different areas of the stool. After placing in MIF fixative the fecal samples were stirred vigorously with the aid of an applicator stick. Upon return to the laboratory at Taipei, the stools were examined as opportunity permitted. Two direct smear preparations (22 x 22 mm coverslip) of several drops each were examined carefully. These samples were withdrawn by pipette from the uppermost layer of particulate sediment. At the time of second processing, one half of the remaining vialed specimen was subjected to MIFC concentration.[2]

SURVEY FINDINGS

Since the period for observation of the people under study was limited, it is difficult to correlate the presence and incidence of intestinal fauna with due regard to ecologic factors and the general habits of the population. On the site, there was a very definite impression that all areas in which studies were made provided ideal conditions for the welfare of parasites of man and lower vertebrates. Table 1 lists the species and incidence for intestinal protozoa and helminths detected in a single fecal sample obtained from people in the Dacca area. No attempt has been made to categorize data by precise age groups, by occupation of families represented, etc., since the numbers of persons from each of three localities is not great.

Most of the intestinal protozoa common to man are represented but the incidence for some species is lower than expected for this area. The total rates for *Entamoeba histolytica* are moderately high and surpass those for *Entamoeba coli* and *Endolimax nana*, usually the predominant species in surveys of this type. Since it is believed that the incidence of pathogenic *E. histolytica* is overestimated in some studies, the larger and small races of *E. histolytica* have been recognized as distinct entities, and therefore listed separately. The small, supposedly non-pathogenic race of *E. histolytica* is much more common than the large form of *E. histolytica* and the ratios of the small to the large race fall in the same range as recorded in similar surveys in Egypt.[3]

The total rates for *Entamoeba histolytica* and the other amebae, alone, indicate that very satisfactory conditions for transmission of parasites exist in the general population of Dacca. Direct person-to-person transmission, perhaps as a rsult of very low household sanitation practices and grossly contaminated food, is suggested by the incidence of *Dientamoeba fragilis*. The general use of water from highly contaminated ponds in and around the city may well account for prevalence of cyst-forming protozoans.

In Pakistan, as in other countries of the Asian-Indian area, it is difficult to estimate the true significance of amebiasis since there is a tendency in statistical reports to indicate morbidity and mortality due to all types of dysentery and diarrheas in a single category. A visit to several hospitals and health institutions in the Dacca area revealed that information on amebiasis was essentially lacking and frequently no attempt was made to differentiate this disease from other similar conditions. When differentiation is considered, determinations are based almost entirely on clinical evidence.

Giardia lamblia is well represented but no higher than expected in a population sample consisting primarily of school age children. There is no adequate explanation for the low incidence or

TABLE I

Incidence (per cent) of intestinal protozoa and helminths in school children in the vicinity of Dacca, East Pakistan*

	Mirpur		Teijgaon		Demra	
	Direct smear	MIFC†	Direct smear	MIFC	Direct smear	MIFC
Protozoa						
Entamoeba histolytica (LR only)‡	6	5	11	6	10	9
Entamoeba histolytica (SR only)	24	19	20	20	25	25
Entamoeba histolytica (LR and SR)	7	4	4	5	4	4
Total *E. histolytica*	37	25	35	31	39	38
Entamoeba coli	37	34	16	20	20	23
Endolimax nana	23	14	25	20	10	9
Iodamoeba bütschlii	18	17	13	6	19	17
Dientamoeba fragilis	9	3	10	15	4	4
Giardia lamblia	16	8	21	20	14	17
Chilomastix mesnili	—	—	—	—	2	2
Trichomonas hominis	1	—	—	1	3	1
Enteromonas hominis	—	—	—	—	2	—
Balantidium coli	1	—	—	—	—	—
Isospora	1	—	—	1	—	—
Helminths						
Hookworm	44	29	52	24	47	13
Ascaris lumbricoides	62	75	36	40	82	83
Enterobius vermicularis	3	1	6	3	1	1
Heterodera	—	—	1	1	1	1
Trichuris trichiura	55	84	39	56	67	87
Acanthocephalan	—	2	—	—	—	—
Diphyllobothrium	1	—	—	—	—	—
Hymenolepis nana	—	—	—	—	1	1
Taenia	1	1	—	—	—	—
Fasciolopsis buski	—	—	—	—	1	1
Haplorchis taichui	—	1	1	1	1	—
Tyroglyphoid mite	5	—	1	—	—	2
Negative for protozoa	19	22	34	39	35	36
Negative for helminths	7	3	26	19	6	6
Negative for both	0	0	0	0	0	0
No. of stools examined	117	117	80	80	98	98

* Given as nearest whole number.

† MIFC (merthiolate-iodine-formalin concentration).

‡ LR = large race *E. histolytica* with cysts measuring more than 10μ; SR = small race with cysts less than 10μ.

absence of other flagellates. *Chilomastix*, a cosmopolitan protozoan reported in most surveys, was detected in only two persons; both were members of the same family. A single stool contained cysts of *Balantidium coli* which is very prevalent in the pigs of East Pakistan, and *Isospora* was recognized in another sample.

Surveys for intestinal fauna of man in East Pakistan are few or lacking. Therefore, there are no figures for comparison of findings for people in the Dacca area with those in other parts of the country. Recently Dutt and Ghosh[4] reported an incidence of 11.3 and 7.5% respectively for *E. histolytica* and *G. lamblia* in a survey in West

Bengal. They also stated that Gupta, *et al.*, in 1954, had reported an incidence of 11.1 and 14.7% of *E. histolytica* and *G. lamblia* respectively in a study of hospital patients at Calcutta which lies 150 miles southwest of Dacca.

Although government health records present in some detail the statistics relating to morbidity and mortality due to snake bites, attacks by wild animals and the more important communicable diseases, no mention is made of helminth parasites. This, probably, is to be expected in a land where the significance of the latter is overshadowed by the more devastating diseases such as malaria, cholera, and smallpox. Upon questioning officials and technicians at hospitals and schools it was learned that helminth infections are considered as "a part of daily living" and that "everybody has worms". Figures presented in Table 1 tend to support this thesis.

It is doubtful that there have been any extensive improvements for the control or reduction of helminth diseases since Chandler's work in this area 30 years ago. *Ascaris* and *Trichuris* are found in the majority of persons examined and hookworm is also common. It is assumed that *Necator americanus* is the prevailing species in the Dacca region but extensive migrations of people in recent years have probably introduced *Ancylostoma duodenale*. It is likely that multiple stool specimens would have demonstrated even greater numbers of infections by worms. As with certain of the protozoa, the rates for incidence of hookworm, *Ascaris*, and whipworm corroborate observations revealing a very low standard of sanitation in the majority of homes. In view of the latter, the very low incidence (1 person) for *Hymenolepis nana* is puzzling. However, Chandler's[5] survey of India showed *H. nana* to be erratic in occurrence and practically absent in some regions. Although it may be argued that the nematodes have much more resistant eggs than *Hymenolepis*, it is not believed that this is the limiting factor for infection by this parasite in Dacca where most environments appear to be optimal for propagation and transmission.

Both *Strongyloides stercoralis* and *Trichostrongylus* were absent although the habits of the people along with their close association with certain animals would seem to allow infection by these worms. Dutt and Ghosh[4] reported a low rate of infection for *Strongyloides* in West Bengal, i.e., 0.4%, and did not list *Trichostrongylus*. Earlier, Chandler found *Trichostrongylus* to be rare in the area now known as East Pakistan, and *Strongyloides* to be very erratic in distribution with a very low incidence in some places.

Since these studies were based entirely upon an examination of fecal samples the rates of infection for *Enterobius* probably are much lower than they would have been had the scotch tape method been employed. In view of the closeness of the people to the soil and the fact that they eat many fresh vegetables, the paucity of *Heterodera* is not readily understood. However, Chandler[5] likewise recorded low rates for this helminth in certain localities. Eggs of an acanthocephalan were found in 2 fecal samples. These possessed the general characteristics of *Macracanthorhynchus hirudinaceus* but have not been listed as such since the measurements were considerably less than given for this parasite. The specific identity of the *Diphyllobothrium* is also questionable since measurements do not agree with those given for diphyllobothriids reported for man. Possibly these represent instances in which the eggs of animal parasites have been consumed with contaminated foods in an enviroment where rats and domestic animals have access to food intended for human use.

Fasciolopsis and *Haplorchis taichui* are the only representatives of the trematoda, although the domestic animals of East Pakistan are heavily infected with *Fasciola* and other helminths. Certainly, the consumption of numerous brackish and freshwater fishes in the Dacca area offers opportunity for greater infection with trematodes than indicated in the present survey.

Concentration of parasites by the use of the MIFC method did not greatly alter the findings for most species. As a matter of fact the rates for a number of protozoa and helminths were higher by the direct smear examination. This is an indication of the efficacy of the MIF vial and direct smear technic by which gradual sedimentation is responsible for considerable concentration of the included organisms and eggs. There are exceptions. In samples from all three schools the rates for *Ascaris* and *Trichuris* were elevated and the eggs of an unidentified acanthocephalan were found as a result of employment of the MIFC technic.

The incidence of intestinal fauna is given for each of three schools from which stool samples were obtained. However, since the living conditions in the different areas were comparable and the overall picture of parasitism similar, it is

unnecessary for additional discussion regarding the findings for each school. It is quite obvious that parasites and related intestinal fauna flourish in the populations studied and their presence reflects the low sanitary standards of people living in an environment where transmission for certain parasites is optimal. All stools contained at least one species of intestinal fauna and only a few escaped infection by helminths. Although helminth diseases elicit little attention, it should be noted that in a cholera ward visited by the author, two children suffered noticeably with intestinal occlusion by *Ascaris* and other patients were anemic as a result of heavy hookworm infection.

SUMMARY

A survey based upon a study of single stool specimens from 300 students in the vicinity of Dacca, East Pakistan, indicates the occurrence of parasites and intestinal fauna in this general area. *Entamoeba histolytica*, *E. coli* and *Endolimax nana* were prevalent, supporting observations indicating that the people, in general, live in an environment which permits effective transmission of certain parasites. The small, non-pathogenic race of *E. histolytica* was much more common than the larger race. *Balantidium coli* and *Isospora* were uncommon with a single infection detected for each. Hookworm, *Ascaris* and *Trichuris* occurred in a high percentage of the population and eggs of *Hymenolepis nana* were found in a single stool. An unidentified acanthocephalan was recorded as well as a single infection with *Fasciolopsis* and *Diphyllobothrium*, and three with *Haplorchis taichui*. All persons studied possessed at least one species of parasite.

ACKNOWLEDGEMENTS

The author is indebted to Dr. Thomas A. Cockburn, ICA, Dacca and to the Ministry of Health of East Pakistan for the use of laboratory and other facilities during the field phases of this study. Dr. Raymond H. Watten, LCDR, MC, USN, Charles Knight, HMC, USN and James E. Reese Jr., HM1, USN, assisted in obtaining information and specimens. G. M. Malakatis, HM1, USN and James E. Reese, Jr., HM1, USN, processed the fecal specimens. Mr. Jalal-Uddin Ahmed, Department of Health served in the capacity of interpreter and as liaison officer for the Mission.

REFERENCES

1. Sapero, J. J., and Lawless, D. K., 1953. The MIF stain-preservation technic for the identification of intestinal protozoa. *Am. J. Trop. Med. & Hyg.*, **2**: 613–619.
2. Blagg, W., Schloegel, E. L., Mansour, N. S., and Khalaf, G. I., 1955. A new concentration technic for the demonstration of protozoa and helminth eggs in feces. *Am. J. Trop. Med. & Hyg.*, **4**: 23–28.
3. Kuntz, R. E., Lawless, D. K., Langbehn, H. R., and Malakatis, G. M., 1958. Intestinal protozoa and helminths in the peoples of Egypt living in different type localities. *Am. J. Tropl Med. & Hyg.*, **7**: 630–639.
4. Dutt, A. R., and Ghosh, A. C., 1955. Incidence of protozoal and helminthic infections of the intestine. *J. Indian M. A.*, **24**: 460–461.
5. Chandler, A. C., 1928. The prevalence and epidemiology of hookworm and other helminthic infections in India. Part XII. General summary and conclusions. *Indian J. M. Res.*, **15**: 695–743.

INTESTINAL PARASITES IN THE PANAMA CANAL ZONE

G. E. COSGROVE*

Gorgas Hospital Laboratory, Ancon, Canal Zone

During the course of two years work (1955–57) at the laboratory of Gorgas Hospital in the Panama Canal Zone, 2500 consecutive fecal specimens were routinely examined for the presence of intestinal parasites. The findings are presented here, as the relative incidence of parasites in patients in the Canal Zone hospitals have not been reported in recent years. The incidence figures are relatively low, probably due to there being a considerable number of American Canal Zone and military personnel and their families included in the hospital census. Approximately 25 years ago several surveys of intestinal parasites were made in rural Panama and in Santo Tomas Hospital in Panama City.[1–5] A much higher proportion of people living in unsanitated or poorly sanitated areas was included in those studies than in the present one.

TABLE 1

Parasite incidence in 2500 fecal specimens

Parasites found	Positive specimens	
	No.	%
One or more species	733	29.3
Giardia lamblia	163	6.5
Entamoeba coli	112	4.5
Entamoeba histolytica	110	4.4
Endolimax nana	82	3.3
Chilomastix mesnili	15	0.6
Iodamoeba bütschlii	14	0.6
Trichomonas hominis	6	0.2
Trichuris trichiura	305	12.2
Hookworm	251	10.0
Strongyloides stercoralis	146	5.8
Ascaris lumbricoides	121	4.8
Enterobius vermicularis	10	0.4
Taenia saginata	4	0.16

MATERIALS AND METHODS

All specimens were examined by zinc sulfate centrifugal flotation and by smears stained with iron hematoxylin. The zinc sulfate technic was performed by suspending about 1 g of feces in tap water in a centrifuge tube, centrifuging, pouring off the supernatant, repeating this step once or twice, finally adding zinc sulfate solution (specific gravity 1.18) to the sediment, mixing and centrifuging. The top 1 cm of fluid was transferred quickly to a new tube, filled with tap water and centrifuged. The sediment was examined. Various other technics such as saline suspensions, water sedimentation, merthiolate-iodine-formalin, and acid-ether-xylene were used as indicated or as checks on routine procedures. The specimens usually were examined on the day received. Approximately half of the specimens were duplicates. All are listed here as separate samples.

RESULTS

The 733 positive samples represent 29.3% of all samples. Two or more different species of parasites were found in 47% of the positive samples; the maximum number found in a single fecal sample was 7. *Entamoeba histolytica* was found in 4.4% of the samples. *Trichomonas hominis* was seen only in saline suspensions of feces and these were not performed routinely. Helminths most frequently found were *Trichuris* (approximately 12%), hookworm (10%), *Strongyloides* (6%) and *Ascaris* (5%). Others were seen only occasionally (Table 1).

DISCUSSION

For several reasons, the incidence of parasites reported here should not be regarded as representative of the Canal Zone population. This included only hospital inpatients and outpatients. Requests for fecal examinations were not routine for all admissions. Many of the samples were duplicates. Examinations were more likely to be requested when parasitic infections were suspected.

Patients at Gorgas Hospital come from home situations ranging from well sanitated to unsanitated. The proportion of individuals admitted from areas with poor sanitation has been markedly reduced in recent years due to restrictive treaty

* Present address: Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee

provisions regarding eligibility for services in this U. S. Government hospital.

About 30 years ago Cort *et al.*[1] reported on hookworm, *Ascaris* and *Trichuris* in inhabitants of rural Panama and urban Panama City. Faust[2] studied strongyloidiasis at Santo Tomas Hospital in Panama City and in native river villages. He also studied the incidence of *E. histolytica* in these groups, in white hospital patients in Gorgas Hospital, and in American clerical personnel in Canal Zone offices.[3] Anderson[4] reported the incidence of *E. histolytica* in Santo Tomas Hospital groups. Comparison of results from these studies and the present one indicates that hookworm infection has been markedly reduced from the former high levels of 86% in rural Panama and 50% in Panama City. In the earlier studies, *Strongyloides* infection was found in 18–20% of the native villagers and Panamanian hospital patients; *E. histolytica* was observed in 13–19% of individuals examined in the Panamanian hospital and in 33–73% of those in various villages but in only 2.8–8.5% of American personnel in the Canal Zone; the incidence of *Ascaris* and *Trichuris* was slightly over 50% in the unsanitated areas.

In 1946, Einhorn and Miller studied helminth infections in pediatric patients at Gorgas Hospital.[5] Their findings are similar to those reported here and are apparently the most recent previous figures on Canal Zone parasite incidence. They divided their patients according to the type of sanitation found in the home community and concluded that helminth infections were found predominantly in children from the unsanitated areas, a relationship which was not examined in our study. It was noted, however, that all instances of heavy multiple parasitism were from unsanitated communities.

Although schistosomiasis, acquired in Puerto Rico or the Antilles, is occasionally encountered in the Canal Zone hospitals, it was not found during the period of this study. Diarrhea and dysentery were common in the series studied here but *E. histolytica* was not a common cause of these conditions.

SUMMARY

Examination of 2500 fecal specimens from ward and outpatients at Gorgas Hospital, Panama Canal Zone, revealed intestinal parasites in about 29%. The most common helminths were *Trichuris trichiura* and hookworm. *Giardia lamblia* was the most common protozoan. *Entamoeba histolytica* was found in about 4%. Approximately half of the various infections were accompanied by one or more others. The incidence figures were based on samples received for routine examination and may not reflect the true incidence of parasites in the Canal Zone population.

ACKNOWLEDGMENTS

I am indebted for the preparation of samples to the medical technologists of Gorgas Hospital laboratory.

REFERENCES

1. Cort, W. W., Stoll, N. R., Sweet, W. C., Riley, N. A., and Schapiro, L., 1929. Studies on hookworm, Ascaris and Trichuris in Panama. *Am. J. Hyg.*, Monogr. Series No. 9, pp. 1–215.
2. Faust, E. C., 1931. Human strongyloidiasis in Panama. *Am. J. Hyg.*, **14:** 203–211.
3. Faust, E. C., 1931. Incidence and significance of infestation with *Endamoeba histolytica* in New Orleans and American Tropics. *Am. J. Trop. Med.*, **11:** 231–237.
4. Anderson, H. H., 1932. Amebiasis in Panama and California with special reference to incidence and treatment. *Am. J. Trop. Med.*, **12:** 459–466.
5. Einhorn, N. H., and Miller, J. F., 1946. Intestinal helminthiasis; clinical survey of 618 cases of infection with common intestinal helminths in children. *Am. J. Trop. Med.*, **26:** 497–515.

THE INGESTION OF BACTERIA AND OF ALTERED BACTERIAL STRUCTURES BY *ENTAMOEBA HISTOLYTICA* IN SHAFFER-FRYE AND IN MODIFIED SHAFFER-FRYE MEDIA*

JAMES G. SHAFFER AND IRIS KEY

The Department of Microbiology and Public Health, The Chicago Medical School, Chicago 12, Illinois

Evidence has indicated that critical factors, essential for propagation of *Entamoeba histolytica*, reside within or upon the bacterial cells used in the Shaffer-Frye (S-F) medium.[1,2] In attempting to get some indication as to how the amebae obtain this material from the bacterial cells, Shaffer, Schuler and Key[3] observed that the bacteria which supported propagation in the S-F medium, produced round bodies under the influence of the penicilin G routinely used in the medium, and that these round bodies were ingested by the amebae. Later, it was reported by Meleney[4] that in a modified Shaffer-Frye medium[5] bacterial cells could be demonstrated within the trophozoites. Meleney utilized the May-Grünwald stain and electron photomicrographs to demonstrate the bacterial cells within the amebae and failed to find the round bodies described by Shaffer *et al.*[3] The bacterial culture used in the modified Shaffer-Frye medium (M-S-F) of Meleney's observations was obtained from this laboratory in 1955 and is a direct descendent of the culture originally used in the S-F medium. The organism used by Shaffer *et al.* was also a direct descendent of this original culture. There were considered to be several possible explanations for the differences in the observations which, in one case showed only ingestion of round bodies and in the other only ingestion of bacterial cells. (1) It was considered possible that the electron miscroscope as used by Meleney might reveal things which might be missed with the darkfield microscope as used by Shaffer *et al.*, or vice versa. (2) Since certain different ingredients and methods are used in the S-F and M-S-F cultural techniques, one or more of these variations might determine what the amebae ingest, or indeed, what is available for ingestion. (3) The bacterial cultures might represent "variants" selected out under somewhat different circumstances, but which reacted differently to penicillin G and the other conditions existing in the cultures.

In order to attempt to determine what was responsible for the variation in observations it was necessary to use both bacterial cultures under similar circumstances. Dr. Meleney very kindly sent a transplant of the bacillus used in his observations and the experiments described in this this report were then done.

MATERIALS AND METHODS

Three bacterial cultures were used. One was the culture received from Dr. Meleney, henceforth referred to as LSU. The other two were those earlier referred to by Shaffer *et al.* as No. 1 and No. 6, and referred to here as S1 and S6. Culture S1 had been used for several years routinely in the S-F medium and had supported excellent propagation of the amebae. Culture S6 had been used routinely some years ago in the S-F medium, but for unknown reasons, had lost its ability to support propagation of the amebae. It was a transplant of this culture which was used in the development of the M-S-F and used by Meleney. At the time the transplant was made this culture was being used routinely in the S-F medium.

All three cultures were established in the fluid thioglycollate medium (BBL No. 136C) of the S-F medium and in the thiomalic acid medium (BBL No. 01-547) used in the M-S-F medium. Each of these was then used to make up the S-F or the M-S-F medium in the routine manner.[1,5]

Observations were first made on each of the bacterial cultures in the S-F and M-S-F media. The media were made in the prescribed manner, but not inoculated with amebae. These observations were made in two ways at intervals between 9 and 24 hours of incubation at 37°C. First, samples were examined by darkfield microscopy, using a Spencer darkfield microscope and, second, samples were placed on a slide previously treated

* This investigation was supported by a grant (E-499C6) from the National Institute of Allergy and Infectious Diseases, Public Health Service.

with Mayer's albumin, dried and stained with methylene blue. Any changes in cellular morphology such as uneven division, unusual motility, increased granularity and the formation of round bodies were noted. Comparisons were made in each case with the various cultures which were handled in a similar manner, but were not exposed to the penicillin G.

Next, cultures were made as above and inoculated with *Entamoeba histolytica* in the routine manner. In all the experiments to be described here the strain of *E. histolytica* used was K-9.* This was considered suitable since other observations had indicated that other large-race strains of *E. histolytica* behave similarly under the circumstances of these experiments.

Samples of the cultures were examined by darkfield microscopy and with appropriate stains to determine what structures, if any, were being ingested by the trophozoites. For the darkfield examination, material was removed by capillary pipette from the area in the bottom of the culture where the amebae were present in greatest numbers. A drop of this material was placed on a clean slide and sealed under a cover slip with petrolatum. In these preparations the trophozoites could be readily seen and studied while still motile.

In order to obtain stained trophozoites for ordinary microscopy and for photography a different procedure was followed: The culture was placed in an International No. 1 centrifuge and spun at 1000 RPM for 10 minutes, after which the culture fluid was removed with a capillary pipette, leaving a volume of approximately 1.0 ml. To this was added 0.4 ml of 10% formalin and thoroughly mixed. Evidence indicated that this resulted in very rapid fixation of the amebae. A drop of this suspension of fixed cells was then placed on a slide which had previously been treated with the thinnest possible layer of Mayer's albumin. The slide was then allowed to dry after which the following staining technique was used, as suggested by Dr. Meleney: (1) Flood slide with May-Grünwald stain (Aloe, Reagent), 5–7 min.; (2) immerse in Giemsa stain (freshly diluted 1:20 in distilled H_2O), 10 min.; (3) rinse in 70% ethyl alcohol, 1 min.; (4) repeat step (3), 1 min.; (5) rinse in acetone, 1 min.; (6) repeat step (5), 1 min.; (7) rinse in acetone-xylol, equal parts, 1 min.; (8) repeat step (7), 1 min.; (9) clear in xylol, 2 min.; (10) repeat step (9), 2 min.; mount in Permount.

* The K-9 strain was obtained from Dr. M. M. Brooke of the Communicable Disease Center, Public Health Service. It was bought from Korea in 1951 by Dr. W. W. Frye.

RESULTS

Observations of the bacterial cultures in the S-F and M-S-F media revealed similar patterns for each culture in the two media. Culture S1 underwent the same series of events described in a previous publication[3] and these changes were identical in the two media. In the first few hours there was uneven cell division with constant bending of the dividing cell and rotation of the short portion during the division process. Some increased granulation could be seen. After about 10 hours there began to appear small, bubble-like structures, sometimes terminal, but more frequently on the side of the bacterial cell (Figure 1c). These structures enlarged until their diameter was about half the length of the bacterial cell. After a time these round bodies appeared free in the medium and their number increased until, after 24 hours, they predominated and there were few bacillary cells remaining. These structures may represent protoplasts, but proof of this is lacking at this time. What eventually happens to the bacterial cell after formation of the round body is still not known.

The LSU and S6 cultures behaved essentially alike in the two media. Here, there was also uneven division of the cells. However, instead of forming round bodies, the cells of these cultures tended to become granular (Figures 1a, b). This granulation could be seen in the darkfield preparations and also was revealed by the methylene blue stain in the fixed preparations. The S6 culture was not as extensively granulated as was the LSU culture. Some cells appeared to have completely lost their cytoplasm and were seen as shell-like structures. This, again, was seen less frequently with the S6 culture.

Observations on the cultures of the amebae were quite interesting. Here, again, results obtained with the two media were, as far as could be seen, identical. With culture S1, no ingested bacterial cells were seen in darkfield preparations, or on the stained preparations either in the S-F or M-S-F media. There was, however, ingestion of the round bodies, first detectable after about 10–12 hours of incubation at 37°C and increasing markedly during the period of observation. There

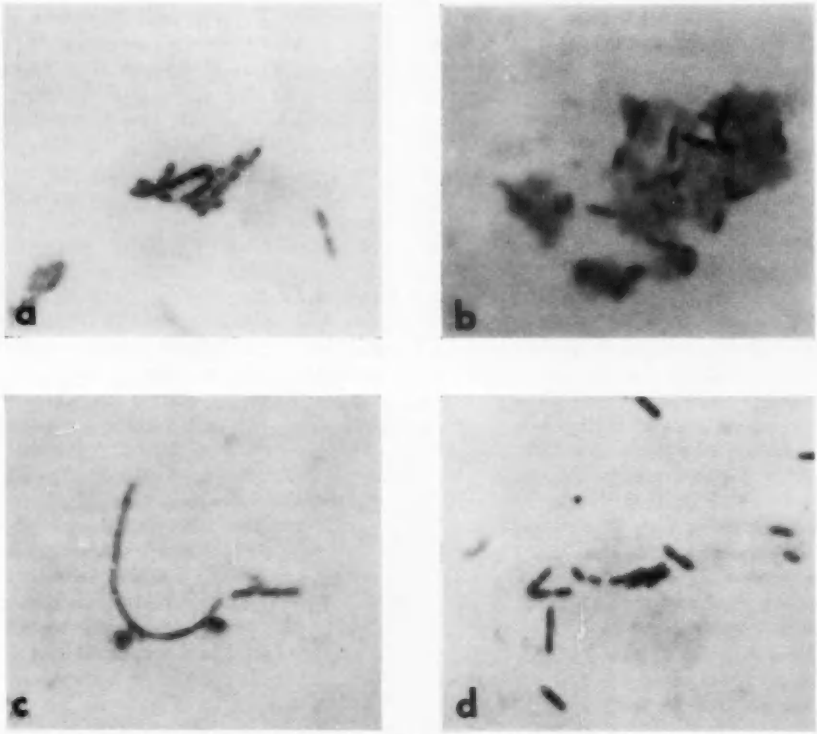


FIG. 1. Methylene blue stains of smears from cultures, LSU, S6 and S1 in S-F medium and in fluid thioglycollate medium. ***a.*** LSU culture in S-F medium (approximately 18 hrs) showing uneven cells and granulation. ***b.*** S6 culture in S-F medium (approximately 18 hrs) showing some uneven cells and some granulation. ***c.*** S1 culture in S-F medium (approximately 18 hrs) showing granulation and round body formation. ***d.*** S1 culture in fluid thioglycollate medium without penicillin G, showing normal cellular morphology. Organisms in all three cultures had identical morphology in the fluid thioglycollate and in the thiomalate medium.

Note: The morphology of the three cultures in the M-S-F medium was identical with that shown in ***a***, ***b*** and ***c***.

was considerable variation in the numbers of round bodies in different trophozoites. In some, no round bodies could be demonstrated while in others these structures were abundant (Figure 2c). As indicated above, this same situation existed in either medium.

The LSU culture also behaved quite similarly in the two media. There was, of course, no round body formation in either medium. However, both darkfield examination and the stained smears did reveal that the trophozoites were ingesting the bacterial cells. In general, the number of ingested bacterial cells was not as great as was the number of ingested round bodies seen when culture S1 was used. There were seldom more than 4 or 5 cells within a single trophozoite (Figure 2a).

The S6 culture yielded results similar to those obtained with the LSU culture, except that it barely supported survival of the amebae in the S-F medium and did little better in the M-S-F medium. As a result, it was more difficult to find trophozoites for observation, and consequently not as many were examined. Nevertheless, when found, the trophozoites tended to have ingested bacterial cells, in the same manner as with the LSU culture (Figure 2b).

Thus, with the S1 culture only round bodies were seen in the *E. histolytica* trophozoites. If the bacterial cells were ingested, they were either missed in the observations or masked by the considerable number of ingested round bodies. With the LSU and S6 cultures, the amebae ingested the bacterial cells, but the numbers ingested were

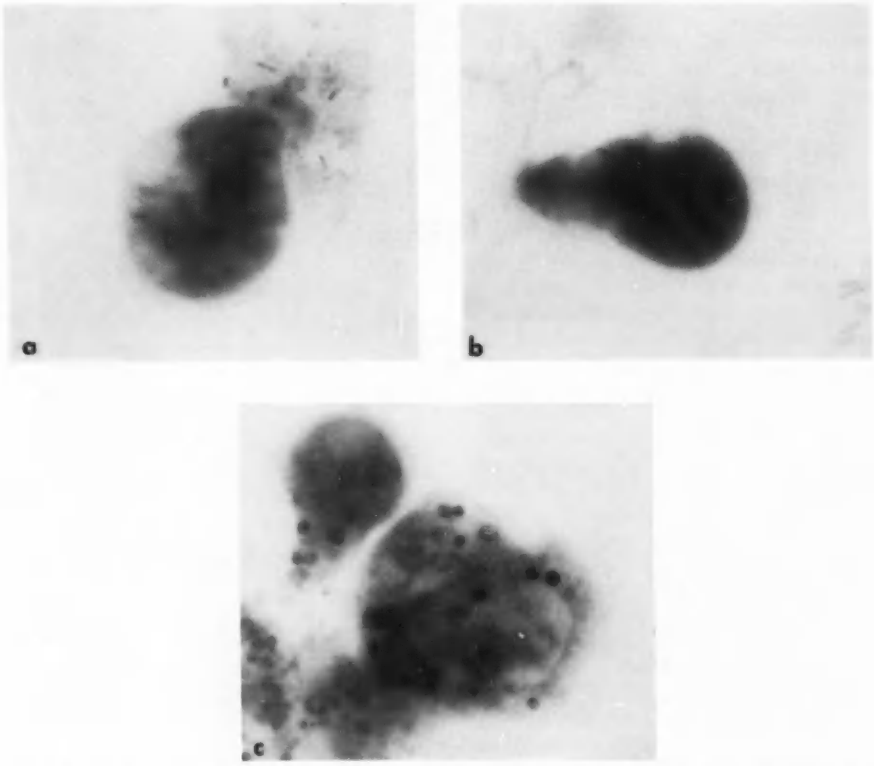


FIG. 2. *Entamoeba histolytica* trophozoites (K-9) in the S-F medium (approximately 24 hrs) showing ingested bacteria or round bodies, May-Grünwald stain × 2500. ***a.*** Trophozoite from culture with LSU bacteria showing ingested bacterial cells. ***b.*** Trophozoite from culture with S6 bacteria showing ingested bacteria. Note that in this preparation the bacteria show more granulation than that seen in Figure 1b. ***c.*** Trophozoites from culture with S1 bacteria showing ingestion of large numbers of round bodies. Note the predominance of round bodies in the material outside the trophozoite. There is one easily discernible bacterial cell in this material and possibly a few others that are difficult to identify.
Note: The appearance in M-S-F medium was in all ways identical with that seen here.

less than the numbers of round bodies ingested when the S1 culture was used.

It would appear then that the differences in the observations of Dr. Meleney and Shaffer *et al.* are the result of variations in the bacterial cultures, rather than differences in the media or the technique.

DISCUSSION

In the S-F medium, as indicated before, evidence indicates that the bacterial cells of the Gram-negative anaerobic bacillus supply critical growth material to the amebae. Presumably, this is also true in the M-S-F medium. This presents at least two questions, (1) what is the nature of this material and (2) how do the amebae get this material from the bacteria. In a previous publication, already referred to[3] it was shown that, in the S-F medium, the amebae ingest the altered bacterial structures (round bodies) produced by the bacteria under the influence of penicillin G. Furthermore, the findings indicated that there was a direct relationship between ability to produce round bodies and ability to support propagation of *E. histolytica.* It was therefore speculated that this might be the way in which the amebae obtained the critical material from the bacterial cells. The observation of Meleney on the ingestion of bacterial cells in the M-S-F medium did not negate the above speculation, since

it could be reasoned that, in a different medium and by being handled differently, the bacterial cells might be susceptible to ingestion by the ameba, without having the cell wall affected sufficiently to produce the round bodies. The amebae could still be getting the necessary materials from the bacterial cells and the ingestion would still be the critical function.

In the present experiments, however, culture S6 was included, since it is related to both S1 and LSU as outlined before, but for unknown reasons does not support propagation of *E. histolytica* in either the S-F or the M-S-F medium. In spite of this failure to support propagation of the amebae, these cells were ingested by them. Evidently these cells did not contain the critical material that the amebae need, or, if they did, the amebae either could not extract or utilize it. Shaffer *et al.*[3] did not report the ingestion of the bacterial cells of culture S6. In fact, these cultures were not extensively studied under the darkfield, since the first examinations were done at 24 hours and the few amebae left at that time were in very poor condition. In the present experiments, the examinations were made between the 9th and 24th hours of incubation and during that period the demonstration of bacterial ingestion could be made.

Thus, the observations that have been reported here would seem to indicate that the ingestion of particles by *E. histolytica* does not necessarily imply that such particles are to be used in whole or in part in the metabolism of the ameba. This would, perhaps, apply not only to bacterial cells, but also to such things as red blood cells and rice grains. Other evidence is necessary before it can be assumed that an ingested particle supplies needed nutrients. It is perhaps more likely that the act of ingestion is determined by physico-chemical characteristics rather than the need for nutrition.

SUMMARY

Three cultural variants of the Gram-negative anaerobic bacillus used in the Shaffer-Frye (S-F) medium for propagation of *Entamoeba histolytica* were studied in the S-F medium and in the modified S-F medium of Reeves, *et al.* Culture S1, which is now used routinely in the S-F medium was found to produce round bodies, probably protoplasts, under the influence of the penicillin G in both media. These bodies were ingested by the amebae during their propagation. At no time were ingested bacterial cells seen with culture S1. Culture LSU, which is used routinely in the modified S-F medium, developed considerable cellular granulation under the influence of the penicillin G, but did not form round bodies in either medium. With this culture, bacterial cells were ingested by the amebae during their propagation. Culture S6, which does not support propagation of *E. histolytica* in either medium, also did not form round bodies in either medium. Nevertheless, the amebae ingested the bacterial cells of this culture. Darkfield microscopy was used for all direct observations in these studies. Permanent preparations were made by fixation of the cultures with 10% formalin followed by staining with the May-Grünwald stain.

Within the limits of these experiments, it appears that the observed changes induced by penicillin G were inherent characteristics of the bacterial cultures and not the result of variation between the S-F and the modified S-F media. The fact that the ameba ingested bacterial cells (LSU and S6) or round bodies (S1) regardless of whether propagation was supported or not, was interpreted as an indication that ingestion of a particle does not necessarily imply that the particle is useful to the ameba.

REFERENCES

1. Shaffer, J. G., 1952. Studies on the growth requirements of *Endamoeba histolytica*. *V*. Studies on the nature of some of the factors in the Shaffer-Frye medium that affect the propagation of *E. histolytica*. *Am. J. Hyg.*, **56**: 119–138
2. Shaffer, J. G., 1952. Factors affecting the propagation of *Endamoeba histolytica in vitro* in the S-F medium and in tissue-bearing substrate. *Ann. N. Y. Acad. Sc.*, **56**: 1033–1047
3. Shaffer, J. G., Schuler, R. W., and Key, I. D., 1958. Studies on the growth requirements of *Entamoeba histolytica*. The ingestion of altered bacterial structures by *E. histolytica* in the Shaffer-Frye medium. *Am. J. Trop. Med. & Hyg.*, **7**: 302–308
4. Meleney, H. E. Personal communication.
5. Reeves, R. E., Meleney, H. E., and Frye, W. W., 1957. A modified Shaffer-Frye technique for cultivating *Entamoeba histolytica* and some observations on its carbohydrate requirements. *Am. J. Hyg.*, **66**: 56–62

REDUCTION IN THE PREVALENCE OF CLINICAL FILARIASIS IN TAHITI FOLLOWING ADOPTION OF A CONTROL PROGRAM

H. N. MARCH, JACQUES LAIGRET, JOHN F. KESSEL AND BEN BAMBRIDGE

Institut de Recherche Médicales de la Polynesie Française, Papeete, Tahiti, and Department of Infectious Diseases, School of Medicine, University of California, Los Angeles, California*

Beye *et al.*[1] reported on the prevalence of clinical manifestations in males in non-periodic Bancroftian filariasis in Tahiti, French Polynesia prior to the institution of a control program. Elephantiasis and microfilarial prevalence were recorded in the regular clinical and blood surveys which included 3390 males in 15 rural districts of Tahiti. In addition seven widely distributed areas throughout the island were set apart for the collection of additional base line epidemiologic data on filariasis and its transmission. In these data, information concerning 203 males examined for clinical manifestations, other than elephantiasis, are included.

In 1954 these districts and areas were consolidated into the island-wide control program described by Kessel.[2] This program was a dual program consisting of mass treatment of the population with diethylcarbamazine and mosquito control by elimination of mosquito breeding places within 100 meters of each dwelling. The mass treatment program consisted of an initial course of 12 monthly doses of 6 mg per kg of body weight of diethylcarbamazine (Notezine) with additional treatments to the few with recurrent positive blood films.

It is considered that data collected by physical examination of the male population for clinical signs and symptoms of filariasis and blood surveys 10 years after the first survey in 1949, and 5 years after the incorporation of the districts and areas into the standardized island-wide filariasis control program, would provide significant data for comparing prevalence rates in these same districts and areas before and after control.

PROCEDURES

In the surveys within the seven small areas, a history of lymphangitis, the presence of enlarged epitrochlear, femoral and inguinal lymph nodes, the presence of hydrocele and of additional signs other than elephantiasis were considered. In both surveys all lymph node enlargement and lymphangitis evidently caused by current bacterial infection were not counted among the cases showing hydroceles, elephantiasis, or lymphangitis considered to be of filarial origin. No effort was made to include the same persons in the second survey that were examined in the first survey. A few migrators have moved into or out of the surveyed areas, but most immigrants to the areas were from islands or districts where a control program had been in operation for at least 2 years and it is considered that they represent a fair cross-section of the island population as a whole.

RESULTS AND DISCUSSION

Table 1 shows the prevalence of clinical filariasis and microfilaremia by age groups among males in 1949 as compared with 1959. Part A of the table is concerned with seven small experimental areas in which clinical filariasis, excepting elephantiasis, was recorded. Part B records prevalence of elephantiasis and microfilaremia in 15 districts of Tahiti, 3390 individuals having been examined in 1949, and 4262 examined for microfilaremia and 7360 for elephantiasis in 1959.

In these surveys, no new cases of elephantiasis or hydrocele were found to have developed in any person after receiving the prescribed course of diethylcarbamazine. This finding together with the loss of elephantiasis cases by natural death in the advanced age groups and their replacement from each preceding age group by people with less elephantiasis have been responsible for the reduced percentages found in each succeeding age group in 1959. Also, some spontaneous regression in clinical findings, notably in early elephantiasis and hydrocele, was apparent among individuals who received treatment during the period. Two new cases, one with

* Aided by gifts to the Pacific Tropic Diseases Project and by grants from the China Medical Board of New York, Inc., and U. S. Public Health Service Grant, #E-392.

TABLE 1

Clinical filariasis and microfilaremia in males

	Age Groups																			
	0–9		10–19		20–29		30–39		40–49		50+		Totals							
Years	1949		1959		1949		1959		1949		1959		1949		1959		1949		1959	
Number examined	47		140		37		115		43		43		32		38		26		41	
Positives	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
A. Clinical filariasis exclusive of elephantiasis in 7 experimental areas																				
Categories																				
Enlarged Nodes Epitrochlear	3	6.38	1	0.7	12	32	2	1.7	16	37	4	9.3	18	56	4	10.5	22	84.5	7	17	12	67	14	28.3	83	40.8	32	7.7
Femoral	4	8.5	0	0	13	35	1	0.87	17	39	5	11.6	14	44	8	21.5	17	65.5	8	19.7	9	50	19	38.7	74	36.4	31	7.2
Inguinal	13	27.6	3	2.1	13	35	10	8.7	13	30	9	20.9	12	37.6	5	13.1	13	50	10	24.3	8	44.3	14	28.3	72	35.4	51	11.9
Hydrocele	0	0	0	0	0	0	0	0	4	9.3	1	2.3	5	15.6	3	7.9	7	27	5	12.2	4	22.2	5	10.2	20	9.8	14	3.2
Other signs excluding elephantiasis:	1	2.1	0	0	3	8.2	0	0	3	6.9	0	0	6	18.7	1	2.6	7	27	1	2.2	3	16.6	2	4.8	23	11.3	4	.93
No signs	28	59.5	136	97.1	13	35	101	87.8	11	25	26	60.4	7	21.9	21	55.2	1	3.8	17	41.4	0	0	20	48.8	60	29.5	321	75.3
History of lymphangitis	0	0	0	0	3	8.2	0	0	7	16	1	2.3	12	37.6	0	0	13	50	2	4.9	11	61.2	4	8.1	47	23.1	7	1.6
B. Elephantiasis and microfilaremia in 15 districts																				
Number examined	751		1264		870		940		549		619		519		529		359		427		344		483		3390		4262	
Microfilaremia	86	11.4	5	0.4	274	31.4	13	1.38	251	45.7	68	11	281	54.1	68	12.8	197	55	54	12.6	196	57	68	14	1285	37.9	276	6.47
Number examined	751		2265		870		1455		549		1010		519		875		359		736		344		999		3390		7360	
Elephantiasis	0	0	0	0	3	0.3	0	0	10	1.8	0	0	43	8.3	12	1.3	72	19.6	39	5.3	110	32	118	11.4	238	7	169	2.24

elephantiasis and one with hydrocele, migrated into the experimental areas during the 10 year period and were treated.

The graphs in Figures 1 and 2 are compiled from Table 1, and record the prevalence of microfilaremia, lymphangitis, elephantiasis and hydrocele, and illustrate decreases that occurred between 1949 and 1959. Elephantiasis and microfilaremial rates are recorded by 10-year age groups. In 1949, 38% of the population of these 15 districts demonstrated a positive microfilaremia while in 1959 only 6.5% were positive. Elephantiasis affected 7% of the total population in 1949 and 2.3% in 1959.

The prevalence of enlarged nodes, lymphangitis, hydrocele and other signs exclusive of elephantiasis, were evaluated by critically examining smaller numbers of people in the same seven experimental areas observed by Beye *et al.* in 1952.[1] Because of the large increase in the numbers examined under 20 years of age in 1959 and the high percentage of negatives in this group, only males above 20 years of age are included in the graphs for lymphangitis and hydrocele. For lymphangitis the results in 1949 and 1959 are 36% and 4% respectively; and for hydrocele in these same years are 17% and 8% respectively. In the seven areas, among 336 males examined under the age of 40 years, which number includes 81 between 20 and 40 years of age, none showed any signs of elephantiasis, while 14 of 90 males above 40 years of age or 15.5% had elephantiasis. Low elephantiasis percentages likewise occurred in the age group under 40 within the 15 districts where 2689 persons examined in 1949 showed 2.1% to have elephantiasis and 5605 examined in 1959 showed only 0.2% to be positive.

In Table 2 several other age groupings are shown for individuals with elephantiasis living within the 15 districts. All demonstrate significant reductions in 1959, but of special interest are first, the group below 20 years of age in which no elephantiasis was found in 1959. The youngest case of elephantiasis encountered in Tahiti was 14 years of age and this was in 1949, when 0.2% of the population under 20 years of age were positive. Now no elephantiasis exists in this age group. Second, in the group above 20 years of age, elephantiasis dropped from 13.2% in 1949 to 5.0% in 1959 which was a 60% reduction. Third, the 20 to 40 age group which is the most active laboring group showed a reduction from

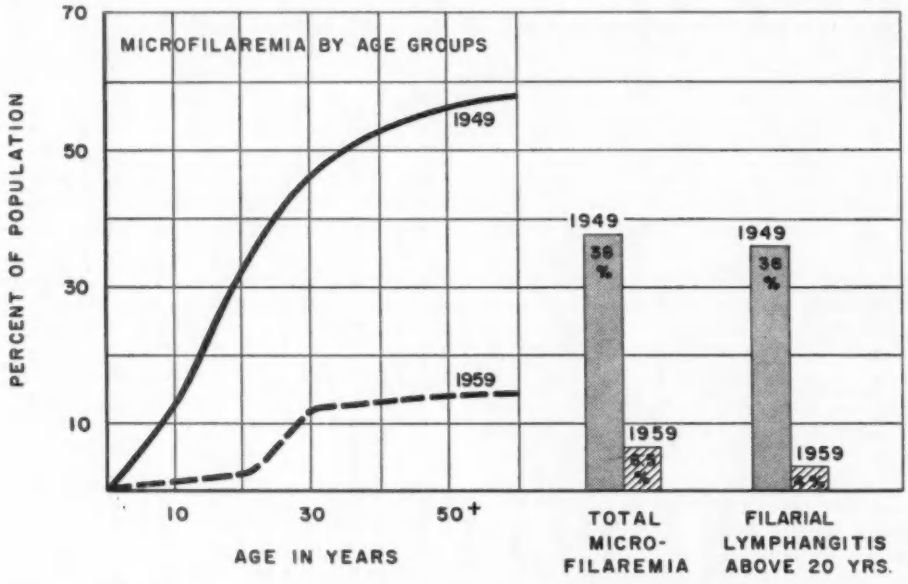


FIG. 1. Prevalence of microfilaremia and lymphangitis in Tahiti in 1949 and 1959

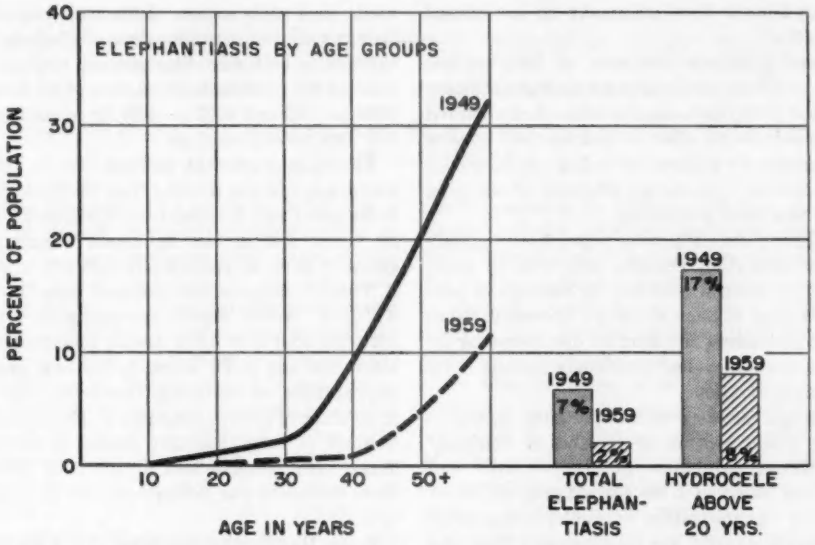


FIG. 2. Prevalence of elephantiasis and hydrocele among males in Tahiti in 1949 and 1959

TABLE 2

Prevalence of elephantiasis in Tahiti

Ages	1949		1959	
	Number examined	Percent positive	Number examined	Percent positive
Below 20 years	1621	0.2	3720	0.0
Above 20 years	1759	13.2	3620	5.0
20–40 years	1068	5.0	1885	0.6
Above 40 years	701	25.5	1735	9.0
Total	3390	7.0	7360	2.3

5% to 0.6%, thus liberating an additional 4.4% of the male population for constructive activities.

Projecting these survey findings to the total rural population of Tahiti, which is approximately 20,000, there would be at least some 500 fewer persons handicapped by elephantiasis in 1959 than there were in 1949. In untreated cases of filariasis, lymphangitis attacks occur several times a year and often last about 3 days, requiring bed rest. Projecting the reduction in lymphangitis found in this survey to the rural population of Tahiti one finds that in 1959 there were some 380 persons above 20 years of age with attacks of lymphangitis in contrast to 3,430 in 1949. It is easy to estimate from the above projections the economic value accruing to the area as a result of the filariasis control program.

Conversely it is also of interest to note in individuals above the age of 20 that the percentage of those free from signs of clinical filariasis increased from 16% in 1949 to 49% in 1959.

In the island of Moorea, where the program was inaugurated in 1956, there is a similar trend in reduction of microfilaremia and clinical symptoms. As no new cases have appeared, it follows that when the present individuals with elephantiasis die, elephantiasis will cease to exist in those islands where an adequate control program is followed.

During the mass treatment with diethylcarbamazine of the Tahitian population, certain observations of value concerning its administration have come to light. Persons experiencing a lymphangitis attack frequently have requested medication voluntarily because they had learned by the personal experiences of relatives and friends that taking diethylcarbamazine prevented further attacks of lymphangitis. This information was spread to other areas and undoubtedly accounted for the fact that when the program was later instituted in

the Isle Sous le Vent practically no one refused medication.

Some individuals who were at first hesitant about accepting medication for filariasis willingly accepted diethylcarbamazine when they observed the anthelminthic effect it had on their own or their neighbor's children by finding adult ascarids in the stools. This visible evidence of the drug action was most convincing.

On Tahiti, some 5% of the population originally refused medication. Personal interviews by members of the central staff usually resulted in persuading most of them to accept treatment. Often they would accept the drug by intramuscular injection when they had previously refused it by oral administration.

Although some workers advocate mosquito control alone for the eradication of filariasis,[3] the marked reductions in clinical signs and symptoms observed in the current program which combines administration of diethylcarbamazine with vector control has demonstrated that this combination is highly satisfactory as a rapid and practical way to control and eventually to eradicate the disease.

Until evidence is presented to show that mosquito control alone can produce equally rapid results or until a more satisfactory chemotherapeutic agent is available, the use of diethylcarbamazine together with mosquito control would appear to be warranted.

SUMMARY

In order to evaluate the filariasis control program in Tahiti, surveys made before and after 5 years of standardized treatment and mosquito control are compared. Both surveys were made on a cross-section of the male population covering clinical manifestations of filariasis. The surveys for both microfilaremia and elephantiasis covered the total male population of 15 districts, 3390 in 1949 and 4262 in 1959 for microfilaremia and 7360 for elephantiasis.

The most important findings are: 1. Overall microfilaremia was reduced from 37.9% to 6.5%. 2. Elephantiasis dropped from 6.9% to 2.2% for all males and in the significant laboring age group of 20 to 40 years it fell from 5% to 0.6%. 3. Total hydroceles were reduced from 9.8% to 3.2%. 4. Acute filarial lymphangitis attacks fell from 36.0% to 4.0% among males surveyed above the age of 20 years. 5. No new cases of elephantiasis or hydrocele developed following application of control measures. 6. Thus a marked decrease in total disability among persons suffering from filariasis was apparent in 1959. 7. Mass treatment was well accepted by the population.

It can therefore be concluded that a combined program of mosquito control and mass chemotherapy with diethylcarbamazine not only greatly lowers the prevalence of microfilaremia but also is followed by marked reductions of the clinical signs of filariasis.

REFERENCES

1. Beye, H. D., Edgar, S. A., Mille, R., Kessel, J. F., and Bambridge, B., 1952. Preliminary observations on the prevalence, clinical manifestations and control of filariasis in the Society Islands. *Am. J. Trop. Med. & Hyg.*, **1**: 637–661.
2. Kessel, John F., 1957. An effective programme for the control of filariasis in Tahiti. *Bull. World Health Organ.*, **16**: 633–664.
3. Iyengar, M. O. T., 1959. Combating filariasis in Polynesia. *So. Pac. Comm. Quart. Bull.*, **9**: 51–55.

USE OF A HEMAGGLUTINATION TEST IN VISCERAL LARVA MIGRANS*

RODNEY C. JUNG AND GUILLERMO PACHECO

Department of Tropical Medicine and Public Health, Tulane University School of Medicine, New Orleans, Louisiana

Eosinophilia may be associated with many kinds of disease, parasitic or non-parasitic in etiology. In some instances typical and readily accessible lesions may provide the pathologist with a diagnosis, as in periarteritis nodosa and Hodgkin's disease. In others the eosinophilia may be the result of an easily detectable parasitic infection. On the other hand, in many cases of eosinophilia an etiologic diagnosis is difficult or impossible to obtain. Included among these are cases of non-patent parasitic infection.

Fortunately not all cases of eosinophilia of unknown origin are alike. Several constant and fairly recognizable syndromes have been described. Loeffler's syndrome, a transient pneumonitis associated with eosinophilia of the peripheral blood, has been shown to occur as the result of not only migration through the lungs by larval nematodes including *Ascaris*, *Ancylostoma* and *Strongyloides*, but also allergy to various non-living substances. It is sometimes observed in periarteritis nodosa. In tropical pulmonary eosinophilia there is persistent pneumonitis, sometimes causing severe symptoms, which responds to treatment with diethylcarbamazine or arsenicals. Recently, evidence has been presented to show that in Malaya this syndrome is caused by infection with filarial worms.[1]

In 1952, Beaver and his co-workers[2] demonstrated that a syndrome of chronic extreme eosinophilia with hepatomegaly in children was caused by tissue infection with larvae of the ascarid *Toxocara*, probably *T. canis*, and suggested for it the term *visceral larva migrans*. They pointed out that the same or similar syndromes had been described many times before under different names and ascribed to other etiologies. More recently it has been recognized that other helminths than *Toxocara* may be responsible.[3] The syndrome consists of marked eosinophilia, hepatomegaly, fever, pneumonitis, and hyperglobulinemia, and occurs most commonly in children between the ages of one and five who habitually eat dirt. The basic lesions are eosinophilic granulomata. Of the clinical features, two appear most important in diagnosis, marked eosinophilia and hepatomegaly. Even hepatomegaly has been found not to occur in mild infections.[4]

At Charity Hospital of Louisiana at New Orleans high eosinophilia accompanied by hepatomegaly is frequently seen, particularly in children, and rarely is an etiologic diagnosis demonstrable. To establish a diagnosis of visceral larva migrans it is necessary to demonstrate the presence of worms in the tissues. Liver biopsy is the only method by which this end is likely to be achieved. Since this procedure carries an inherent though slight risk, investigations were undertaken for the development of an immunologic test for the diagnosis of non-patent helminth infections such as larval toxocariasis. A reliable test of this kind would allow the clinical and epidemiological features of the several varieties of these infections to be delineated.

Since experiments indicated that intradermal tests were not likely to provide adequate information for diagnosis[5, 6] a serologic test was sought. Because of the reported successful use of an indirect hemagglutination technic in the diagnosis of helminthic infections,[7, 8] and of ascarid infections in particular,[9, 10] this type of test was applied to the problem. The procedure used is derived from the test of Middlebrook and Dubos[11] with modifications by Boyden,[12] Stavitsky[13] and Kagan.[7]

MATERIALS AND METHODS

Hemagglutination Test. Sheep cells, stored in Alsever's solution were washed 3 times with 5 to 6 volumes of phosphate buffered saline solution at pH 7.2 (hereafter referred to as P.B.S.). The washed cells were suspended in P.B.S. at 2.5% concentration. For tanning, 1 volume of cell suspension was mixed with an equal volume of 1:20,000 tannic acid (Baker, N. F.), prepared freshly each day, and incubated

* Supported by Grant E-1159 of the National Institutes of Health, U. S. Public Health Service.

at 37°C for 10 minutes. The tanned cells were washed once with P.B.S. and sensitized by adding 3 to 4 volumes of antigen to 1 volume of packed cells. This was mixed and incubated at room temperature for 15 minutes after which the suspension was stabilized by washing with 1:100 normal rabbit serum (N.R.S.). After the final washing the cells were diluted with 1:100 N.R.S. to a 2.5% suspension, which was then used in the test.

All of the sera used in the test were inactivated at 56°C for 30 minutes and two-fold serial dilutions were made using 1:100 N.R.S. as diluent, starting with 1:10 dilution.

One drop, approximately 0.05 ml, of cell suspension was added to each tube. The readings were made according to Salk's method,[14] that is, a layer of cells spread evenly across the bottom of the tube in a blanket was considered positive; a button of cells without ragged edges negative.

Controls for the test were as follows: (a) diluent control consisting of diluent (1:100 N.R.S.) and tanned, sensitized cells; (b) tanned cells control consisting of known positive serum and tanned, unsensitized cells; (c) normal serum control consisting of normal serum and tanned sensitized cells; (d) known positive control consisting of a known positive serum plus tanned, sensitized cells. All of the controls except the last had to be negative before the test was considered valid.

Preparation of Antigens. Adults of *A. lumbricoides* and of *T. canis* were washed, then emulsified with a blendor in P.B.S. at 5°C for 5 minutes and the emulsion stirred overnight at 5°C in order to complete extraction of the soluble substances, then filtered through gauze to remove large particles that remained, in most cases consisting of cuticle. The filtrate, designated whole worm extract, was precipitated with an equal volume of chilled 95% ethanol. The mixture was allowed to stand for approximately 5 hours at 5°C after which it was centrifuged at 850 X G for 30 minutes. The precipitate was dissolved in P.B.S. and the solution centrifuged to remove insoluble particles. The solution was again treated with half its volume of alcohol and allowed to stand at 5°C for approximately 4 hours after which it was centrifuged as above. The precipitate was redissolved in P.B.S., the solution centrifuged to remove insoluble particles and the supernate dialyzed against running tap water for 24 hours and 4 changes of distilled water at 5°C for 48 hours; then distributed into ampules and lyophilized.

The preparation of this fraction differs from that of the ethanol-precipitated fraction of Kagan *et al.*[15] mainly in that the extract is not boiled or autoclaved.

The resulting antigens were found to give positive biuret, Millon and Molisch tests. Block titrations of the antigens against known positive rabbit sera gave an optimal antigen concentration which has been used in all subsequent routine tests. At optimal concentration the *T. canis* antigen was found to contain 0.6 μg/ml of nitrogen (method of Kabat and Mayer[16]) and 0.9 μg/ml of carbohydrate (method of Neish[17]). The *A. lumbricoides* antigen was found to contain 9 μg/ml of nitrogen and 60 μg/ml of carbohydrate.

Sources of Sera. The human sera tested were obtained from individuals of several categories as follows:

(1) Clinical cases of eosinophilic disease, including some suspected of being visceral larva migrans.
(2) Contacts of cases, particularly siblings, playmates and parents.
(3) Cases of known infection with parasites other than *Toxocara.*
(4) Siblings and parents of (3).
(5) Individuals not known to have parasitic infection or eosinophilia.

Sera were obtained from patients mainly at the Charity Hospital of Louisiana at New Orleans, Lallie Kemp Hospital, Independence, La., and the Tulane Tropical Medicine Clinic. In addition, specimens have been received from cases in various parts of the United States and several foreign countries.

Persons included in the analysis had physical examinations, differential blood counts and stool examinations. Chest X-ray and additional clinical laboratory studies were done on many others. For purposes of analysis, hepatomegaly and eosinophilia exceeding 30% were arbitrarily taken as clinical criteria of visceral larva migrans.

RESULTS

a. *Negative Controls.* Of approximately 500 individuals from whom serum was collected 51 were selected solely on the following criteria and designated as "negative" controls: They did not have eosinophilia, hepatomegaly, or demon-

strable intestinal helminthic infections. Persons who met these criteria but who were members of families of cases of visceral larva migrans or of persons who were being studied as parasitized controls were excluded.

Table 1 shows the titers with *Ascaris* and *Toxocara* antigens obtained with sera of these 51 persons. It is apparent in this frequency table that the titers were predominantly below 1:160 with both *Ascaris* and *Toxocara* antigens. Only two individuals had *Ascaris* titers of 1:160 or above and none had *Toxocara* titers above 1:80. On the basis of the above distribution, titers of 1:80 and below have been considered valueless for diagnosis of visceral larva migrans. Titers of 1:160 and above have tentatively and arbitrarily been taken to be indicative of infection with *Ascaris* or *Toxocara* larvae. Of the negative controls 96% had titers of 1:80 or below (Table 2).

TABLE 1

Numbers of sera with indicated reciprocals of titers of hemagglutination reactions from persons without eosinophilia, hepatomegaly or intestinal parasitosis and with no known contact with VLM cases

	Toxocara antigen						Total
	0	10	20	40	80	160	
Ascaris antigen							
0	30		1				31
10	1		3				4
20	3		1	1	1		6
40	3	3					6
80	1		1				2
160				1			1
1280	1						1
Total	39	3	6	2	1		51

b. *Contact Controls.* One hundred thirty-nine individuals were studied who were without eosinophilia and hepatomegaly but who were siblings or close contacts of cases. These were designated "contact controls." Of these, 127 (91.4%) gave titers less than 1:160 with both *Ascaris* and *Toxocara* antigens. Serum from one (0.7%) reacted in high titer with *Toxocara*, from 10 (7.2%) with *Ascaris* antigen and from one with both *Ascaris* and *Toxocara* antigens (Table 2).

c. *Cases of Visceral Larva Migrans.* Twenty-nine persons had eosinophilia over 30% and hepatomegaly. In general, they presented the features, so far as is now known, of visceral larva migrans. The following exceptions must be noted: Not all of the cases were observed for a long enough period to establish another important criterion of visceral larva migrans, chronicity. One individual followed a downhill course and eventually

TABLE 2

Results of hemagglutination tests with Ascaris *and* Toxocara *antigens and sera from persons of various categories*

Category	No. of persons in category	Titers 1/80 or lower		Titers 1/160 or higher							
		With *Ascaris* and *Toxocara* antigens		With *Ascaris* but not *Toxocara* antigen		With *Toxocara* but not *Ascaris* antigen		With both *Toxocara* and *Ascaris* antigens		With either or both antigens	
		No.	%	No.	%	No.	%	No.	%	No.	%
Visceral larva migrans*	29	6	20.7	5	17.2	2	6.9	16	55.2	23	79.3
Negative controls	51	49	96.1	2	3.9	0	0	0	0	2	3.9
Contact controls	139	127	91.4	10	7.2	1	.7	1	.7	12	8.6
Ascariasis with or without other helminth infection	58	48	82.8	9	15.5	1	1.7	0	0	10	17.2
Ascariasis, no other helminth infections	23	20	87.0	3	13.0	0	0	0	0	3	13.0
Strongyloidiasis	35	17	48.6	11	31.4	0	0	7	20.0	18	51.4
Ancylostomiasis	14	13	92.9	1	7.1	0	0	0	0	1	7.1
Trichuriasis	48	39	81.3	8	16.6	1	2.1	0	0	9	18.7

* Diagnosed on clinical basis—hepatomegaly, eosinophilia exceeding 30%.

died, apparently of exhaustion. Such a course of gradual decline has not been reported in visceral larva migrans, although death may occur of acute respiratory failure. Furthermore, although microscopic granulomata were demonstrated at autopsy, no larvae could be found even by digestion of the entire liver and lungs.

The results obtained with hemagglutination tests in these cases using *Ascaris* and *Toxocara* are summarized in Table 2. Sera from only six (20.7%) had titers with both *Ascaris* and *Toxocara* antigens below 1:160. One of them was the above mentioned patient who apparently did not have visceral larva migrans in spite of the presence of many of the clinical features of that disease.

d. *Persons Recovered from Visceral Larva Migrans.* There were five patients who had had typical syndromes of visceral larva migrans from 3 to 7 years previously and who had been followed as out-patients since then. In all the liver size and eosinophilia had subsided to such a degree that they would no longer fulfill the clinical criteria of visceral larva migrans and therefore have not been included with the 29 cases described above. In none of these cases could antibody against *Toxocara* or *Ascaris* be demonstrated.

e. *Other Eosinophilic Disease.* Many other patients had eosinophilic disease which did not appear to correspond to visceral larva migrans and so have not been included in that group. The following are examples:

(1) *Eosinophilia with lymphadenopathy.* Two patients, one a 2-year-old white boy and the other a 50-year-old colored female, had eosinophilia above 30% and cervical and axillary lymphadenopathy. When lymph node biopsies were examined a chronic inflammatory pattern was found in the former, an eosinophilic abscess in the latter. In the boy, hemagglutination titers of 1:320 were obtained with *Ascaris* antigen and 1:20 with *Toxocara* antigen. The *Ascaris* titer subsided within a year. An *Ascaris* titer of 1:160 and *Toxocara* of 1:320 were obtained with serum from the colored woman with eosinophilic lymphadenitis.

(2) *Eosinophilia with subcutaneous abscess.* One patient, an English boy, had 32% eosinophilia, a normal liver and a sterile subcutaneous abscess of the thigh. His serum produced an initial titer with *Ascaris* antigen of 1:5120 but a month later was only 1:160. His titer with *Toxocara* antigen was 1:40.

(3) *Eosinophilia with granulomatous iritis.* One patient, a 2-year-old white boy, had a massive eosinophilia with granulomata in one iris. He was a habitual dirt-eater, and *Toxocara* eggs were found in dirt from sites at which he had been frequently noted indulging in this practice. A puppy at his home was infected with *Toxocara.* The boy did not exhibit features of visceral larva migrans other than eosinophilia. Maximum titers of 1:160 were obtained with *Ascaris* and 1:80 with *Toxocara* antigens.

(4) *Loeffler's syndrome (Transitory eosinophilic pneumonitis).* There were five individuals who had eosinophilia and pneumonitis. All of these patients had asthmatic type of dyspnea at the time of admission to the hospital. The eosinophilia, though above normal in all, was above 30% in three and below 30% in two. One patient had an enlarged liver. A high hemagglutination titer with *Toxocara* antigen was obtained in one instance. Titers with *Toxocara* antigen in the other cases and with *Ascaris* antigen in all cases were low.

f. *Intestinal Parasitic Infections*

(1) *Ascariasis.* Fifty-eight persons who had intestinal ascariasis but lacked eosinophilia and hepatomegaly were tested. In 23 of these patients, *Ascaris* was the only intestinal helminth; whereas, in the other 35 patients other helminths were found. Table 2 summarizes the results in the former group. There was no cross reaction between *Toxocara* antigen and the serum from patients with *Ascaris* infection alone; serum from only one mixed infection gave a titer of 1:160 or over with *Toxocara* antigen. On the other hand sera from 13% of the persons with pure *Ascaris* infection and 15.5% of the mixed infections gave titers of 1:160 or over with *Ascaris* antigen. Over 80% of the entire group had titers under 1:160 with both antigens.

(2) *Strongyloidiasis.* Of 35 persons with strongyloidiasis 17 (48.6%) had low titers with both *Ascaris* and *Toxocara* antigens (Table 2). Seven (20%) had high titers with both *Ascaris* and *Toxocara* antigens and 11 with *Ascaris* antigen only. There was thus a higher degree of cross-reaction with the *Strongyloides* infected than with any other groups of controls (Table 2).

(3) *Hookworm infection.* Of 14 patients with hookworm infection and without features of

visceral larva migrans 13 had low titers with both *Ascaris* and *Toxocara* antigens (Table 2). One serum had a high titer with *Ascaris* antigen and did not react with *Toxocara* antigen.

(4) *Trichuriasis*. Of 48 patients with whipworm infection, 39 (81.3%) had low titers with both *Toxocara* and *Ascaris* antigens (Table 2). Only one serum gave a high titer with *Toxocara* antigen; 8 gave high titers with *Ascaris* antigen.

Statistical Analysis

The results of hemagglutination tests in the negative controls were not significantly different from those in the contact control group ($\chi^2 = 1.33$; $0.368 < p < 0.607$). On the other hand the difference between results with tests on serum from cases of visceral larva migrans and from negative controls and contact controls was highly significant ($\chi^2 = 107$).

DISCUSSION

The data here presented indicate that the hemagglutination test may provide useful evidence for the diagnosis of visceral larva migrans. Although sera from patients with visceral larva migrans proved by demonstration of larvae in biopsy specimens have not been available, sera from cases selected on clinical grounds have predominantly had high titers when tested with *Ascaris* and/or *Toxocara* antigens in contrast to the controls. Furthermore, cross-reaction in patients with known intestinal parasitic infection was slight except in the case of strongyloidiasis. Nevertheless, several observations require clarification and decrease the confidence with which the test can be applied.

In the cases with clinical features of visceral larva migrans more individuals (21) had titers over 1:80 against *Ascaris* than against *Toxocara* (18). This may be due to cross-reaction between the two antigen-antibody systems, and the predominance of individuals with high titers against both antigens is evidence in favor of this view. The presence of common antigens in *Ascaris* and *Toxocara* has been suggested by the work of Kagan.[18] On the other hand the fact that five individuals had high titers against *Ascaris* and low titers against *Toxocara* suggests that a syndrome resembling visceral larva migrans may be produced in some cases by *Ascaris* rather than *Toxocara* larvae. This would be theoretically possible if infective *Ascaris* eggs are frequently ingested by an individual in large numbers even though *A. lumbricoides* larvae are capable of surviving in tissues only 2 or 3 weeks[19–21] as compared with *Toxocara* larvae which may live many months.[3, 4, 20] This has been previously suggested.[22]

Since most of the cases of visceral larva migrans in the study were infants only small amounts of serum were available. Cross absorption studies such as have been carried out by others[9] to demonstrate specific antibody to *Ascaris* or *Toxocara* infections in experimental animals could not be carried out with such small quantities of serum.

Over 20% of clinical cases of larva migrans were found to have low titers against both *Ascaris* and *Toxocara* (under 1:160). Failure to demonstrate high antibody titers in these instances may be explained in several ways. The signs and symptoms of visceral larva migrans may be produced by tissue parasites other than those for which the sera had been tested. For example *Capillaria hepatica* is known to produce a markedly similar picture[23]; *Strongyloides* may duplicate some of the findings, and experiments by Nichols[24] suggested that larvae of *Ancylostoma caninum* may possess invasive and survival properties equaling those of *T. canis*. Furthermore it is even possible that the eosinophilia and hepatomegaly may be produced in the absence of any helminth.

Visceral larva migrans is characterized by a chronic course. Eosinophilia may persist for as long as 2 years. Not all of our cases were followed long enough to establish the fact of chronicity. Therefore some may not have had visceral larva migrans but a more transient disease, such as Loeffler's syndrome or some as yet unnamed eosinophilic syndrome. This may explain the absence of *Toxocara* antibodies in some sera.

Low titers in several instances were probably the result of a single specimem of serum being taken either too late or too early in the illness. Serial testing shows that titers may fluctuate relatively rapidly. The value of a single specimen is therefore reduced.

High hemagglutination titers in normal individuals may be the result of hidden or subclinical *Toxocara* infection rather than cross-reaction. Masking of the eosinophilia of toxocariasis could especially readily occur in individuals with strongyloidiasis since the latter infection itself

characteristically provokes an eosinophilia of considerable degree.

Concurrent infection with ascarid larvae may be the cause of the considerable frequency with which *Ascaris* and *Toxocara* antigens reacted in high titer with sera of patients with strongyloidiasis. However, since there is no apparent reason for individuals infected with *Strongyloides* to have concurrent larval ascarid infection more frequently than the other groups studied, high titers in those with strongyloidiasis are more probably due to cross-reaction.

The concurrence of high hemagglutination titers against *Ascaris* and *Toxocara* antigens with clinical visceral larva migrans and subsidence of these titers even though an intestinal *Ascaris* infection remains suggests that the test may be detecting some acute-phase substance, stimulated by the host reaction but not specific for the etiologic agent. Since *Strongyloides* through hyperinfection may produce a systemic eosinophilic reaction due to larvae migrating through the tissues, it and larval toxocariasis may both perhaps stimulate the production of the same hypothetical acute-phase substance. Studies involving serum fractionation to determine the nature of the visceral larva migrans antibody are under way.

In cases of eosinophilic disease without features corresponding to the syndrome of visceral larva migrans, results with the hemagglutination test were variable. No conclusive evidence that they represent variants of visceral larva migrans was obtained, although in the case of granulomatous iritis the history of dirt-eating and presence of *Toxocara* eggs in the soil strongly suggest that the patient had larval toxocariasis.

SUMMARY

A hemagglutination test using tanned sheep erythrocytes was used to test serum of persons suspected of having visceral larva migrans, negative controls, contacts of suspected cases, and persons infected with various intestinal parasites. Cases of visceral larva migrans selected by clinical criteria usually (79.3%) showed titers for either *Toxocara* and/or *Ascaris* antibody above 1:80. Sera from over 90% of negative controls and contacts without signs of visceral larva migrans had low titers (1:80 or lower) with both antigens. There was little cross-reaction between sera from patients with hookworm, whipworm and visceral larva migrans, but sera from patients with strongyloidiasis frequently gave high titers with *Toxocara* antigen (20%) and with *Ascaris* antigen (51.4%). Patients having intestinal ascariasis but no signs of visceral larva migrans had low titers with *Ascaris* and *Toxocara* antigens.

REFERENCES

1. Danaraj, T. J., da Silva, L. S. and Schacher, J. F., 1959. The serological diagnosis of eosinophilic lung (tropical eosinophilia) and its etiological implications. *Am. J. Trop. Med. & Hyg.*, **8**: 151–159.
2. Beaver, P. C., Snyder, C. H., Carrera, G. M., Dent, J. H. and Lafferty, J. W.. 1952. Chronic eosinophilia due to visceral larva migrans. *Pediatrics*, **9**: 7–19.
3. Beaver, P. C., 1956. Larva Migrans. *Exper. Parasitol.*, **5**: 587–621.
4. Smith, Margaret H. D. and Beaver, P. C., 1953. Persistence and distribution of *Toxocara* larvae in the tissues of children and mice. *Pediatrics*, **12**: 491–497.
5. Jung, R. C. and Pacheco, G., 1958. The use of intradermal and indirect hemagglutination tests for the diagnosis of visceral larva migrans. *Proc. VI Internat. Cong. Trop. Med. & Mal.* (in press).
6. Jung, R. C. and Pacheco, G., 1958. Relationship of clinical features to immunologic reactions in visceral larva migrans. *Am. J. Trop. Med. & Hyg.*, **7**: 256.
7. Kagan, I. G., 1955. Hemagglutination after immunization with schistosome antigens. *Science*, **122**: 376–377.
8. Garabedian, G. A., Matossian, R. M. and Djanian, A. Y., 1957. An indirect hemagglutination test for hydatid disease. *J. Immunol.*, **78**: 269–272.
9. Kagan, I. G., 1958. Hemagglutination tests with *Ascaris* antigens. *J. Immunol.*, **80**: 396–399.
10. Kagan, I. G., Norman, L. and Allain, D., 1959. Studies in the serology of visceral larva migrans. I. Hemagglutination and flocculation tests with purified *Ascaris* antigens. *J. Immunol.*, **83**: 297–301.
11. Middlebrook, G. and Dubos, R. J., 1948. Specific serum agglutination of erythrocytes sensitized with extracts of tubercle bacilli. *J. Exper. Med.*, **88**: 521–528.
12. Boyden, S. V., 1951. The absorption of proteins on erythrocytes treated with tannic acid and subsequent hemagglutination by antiprotein sera. *J. Exper. Med.*, **93**: 107–120.
13. Stavitsky, A. B., 1954. Micromethods for the study of proteins and antibodies. I. Procedure and general applications of hemagglutination and hemagglutination-inhibition reactions with tannic acid and protein-treated red blood cells. *J. Immunol.*, **72**: 360–367.
14. Salk, J., 1944. A simplified procedure for titrating the hemagglutination capacity of influenza virus and the corresponding antibody. *J. Immunol.*, **49**: 87–98.
15. Kagan, I. G., Jeska, E. L. and Gentzkow, C. J., 1958. Serum-agar double diffusion studies with *Ascaris* antigens. *J. Immunol.*, **80**: 400–406.

16. KABAT, E. A. AND MAYER, M. M., 1948. *Experimental Immunochemistry*, p. 287. Springfield, Ill., Charles C Thomas.
17. NEISH, A. C., 1952. *Analytical Methods for Bacterial Fermentations*, 2nd Revision, p. 33. National Research Council of Canada, Report No. 46-8-3.
18. KAGAN, I. G., 1957. Serum-agar double diffusion studies with *Ascaris* antigens. *J. Infect. Dis.*, **101**: 11–19.
19. HOEPPLI, R., FENG, L. AND LI, F., 1949. Histological reactions in the liver of mice due to larvae of different *Ascaris* species. *Peking Natl. Hist. Bull.*, **18**: 119–132.
20. SPRENT, J. F. A., 1952. On the migratory behavior of the larvae of various Ascaris species in white mice. I. Distribution of larvae in tissues. *J. Infect. Dis.*, **90**: 165–176.
21. SPRENT, J. F. A., 1953. On the migratory behavior of the larvae of various Ascaris species in white mice. II. Longevity of encapsulated larvae and their resistance to freezing and putrefaction. *J. Infect. Dis.*, **92**: 114–117.
22. JUNG, R. C., 1954. The predominance of single-brood infections in human ascariasis. *J. Parasitol.*, **40**: 405–407.
23. OTTO, G. F., BERTHRON, M., APPLEBY, R. E., RAWLINS, J. C., AND WILBUR, O., 1954. Eosinophilia and hepatomegaly due to *Capillaria hepatica*. *Bull. Johns Hopkins Hosp.*, **94**: 319–336.
24. NICHOLS, ROBERT L., 1956. The etiology of visceral larva migrans. II. Comparative larval morphology of *Ascaris lumbricoides*, *Necator americanus*, *Strongyloides stercoralis* and *Ancylostoma caninum*. *J. Parasitol.*, **42**: 363–399.

HUMAN SPARGANOSIS IN MISSISSIPPI: REPORT OF TWO CASES

T. J. BROOKS, JR., W. F. HUTCHISON, T. J. SAFLEY, AND T. G. ROSS

University of Mississippi Medical Center, Jackson, Mississippi

On March 7, 1933, Mueller[1] autopsied two cats from the city of Syracuse, New York, and found in them a new species of *Diphyllobothrium*. He named the worm *Diphyllobothrium mansonoides*, and published a description of the parasite. A principal characteristic of the worm was the lateral position of the terminal uterine chamber. In later work, Mueller[2] showed that the water snake *Natrix sipidon* was the normal host for the plerocercoid stage.

In 1929 Faust, Campbell, and Kellogg[3] had suggested the subgenus *Spirometra* for those species with a coiled outer uterus as opposed to the rosetted outer uterus of the sub-genus *Diphyllobothrium*, and in 1937 Mueller[4] raised the name *Spirometra* to generic status.

By 1937 the adult worm had been found in the house cat in New York, the bobcat (*Lynx rufus*) in North Carolina, the bobcat (*Lynx rufus floridanus*) in Florida, and the house cat in Louisiana[5] and it was suggested[6] that this species should be considered as a probable cause of human sparganosis in North America.

Human sparganosis was first observed by Manson[7] in 1882 at the autopsy of a native of Amoy, China. The adult of this worm has long been known in the Orient as *Diphyllobothrium mansoni*.

The first case of human sparganosis in North America was a case of *Sparganum proliferum* recorded by Stiles[8] in 1908 in a man in Florida. In 1914 Moore[9] reported what he termed "the first reported American case" of *Sparganum mansoni*. The worm had been surgically removed by him in 1911 from the left breast of a male patient, aged 47 years, who had been a resident of Florida and Texas. Identification of the parasite was made by B. H. Ransom.

By 1958 it was stated by Botero and Gomez[10] that in addition to these two "about 10 more cases have been described from the United States." According to these authors only three cases have been reported from the South American continent. Owing to its infrequent observation in the Western Hemisphere, the following two cases are of interest.

CASE HISTORIES

Case 1. J. C. C., a 54-year-old white female, was admitted to the Mississippi Baptist Hospital on March 24, 1953, with a mass in the right breast. The lesion had been present for about 6 months. It was painless, and had not been associated with any changes in menses.

The patient stated that this lump first appeared several years previously as a small mass in the right arm. From time to time it moved about and occasionally disappeared only to reappear at another site higher on the extremity. It finally disappeared altogether and reappeared in its present location in the breast. There was no history of trauma.

The past history was otherwise non-contributory. The patient had travelled extensively throughout the U. S. during the time her husband was in the Navy, but she had never been outside the continental limits of the United States.

Physical examination revealed a mass measuring 2.5 x 1.5 cm situated in the outer quadrant of the right breast. It was firm and appeared to be attached to the skin. A chest roentgenogram was negative as were accessory clinical data.

Under local anesthesia the mass was surgically excised, and on frozen section was found to contain a larva. The material was then preserved and was later identified as a sparganum by Dr. K. M. Heard, Pathologist at the Mississippi Baptist Hospital. It was subsequently sent by him to Dr. P. C. Beaver, who confirmed this diagnosis and stated, "We presume most of the specimens taken from humans are *Spirometra mansonoides* but we are not justified in stating that they actually are that species or even that genus."

Case 2. G. M. V., a 62-year-old housewife, was seen on May 29, 1957, complaining of a small nodule on the right anterior chest. The lesion had been present for 3 or 4 months. It had not been painful, although at times it had caused some discomfort. There was no other significant history.

On further questioning the patient stated that that she had never been outside the State of

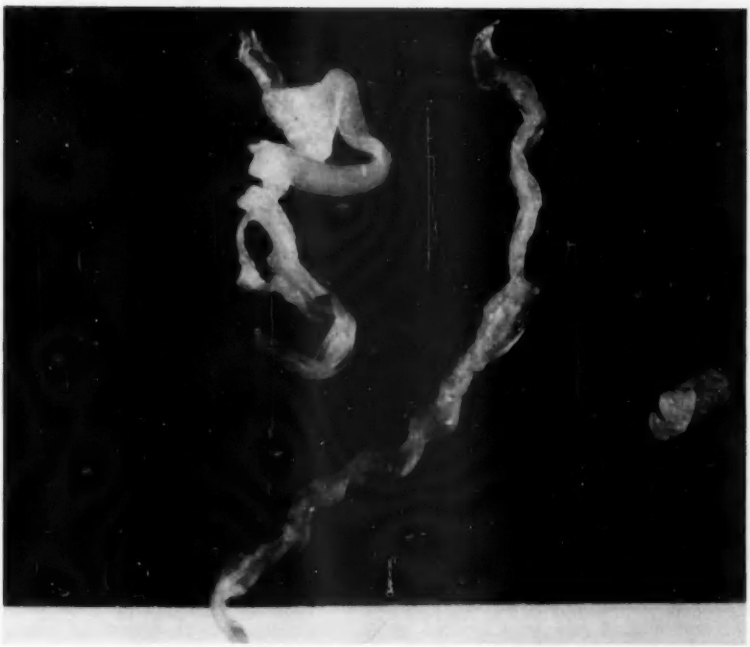


FIG. 1. Fragments of the sparganum removed from the anterior chest wall of patient No. 2. The longest segment was about 13 mm after fixation.

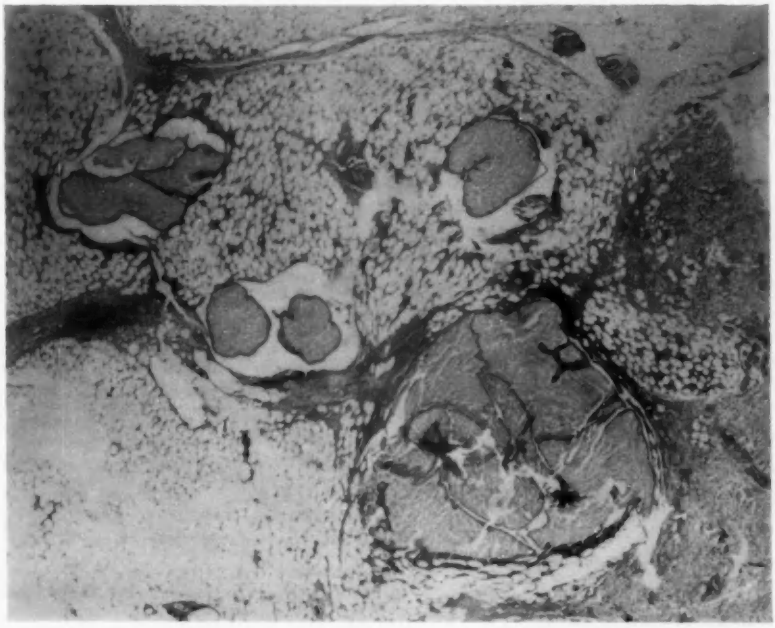


FIG. 2. Cross sections of the sparganum removed from patient No. 2. (× 18)

Mississippi except on one visit to Montgomery, Alabama. She denied the use of meat poultices.

On physical examination there was found to be present a firm lesion approximately 1 cm in diameter situated about 5 cm below the lower border of the right breast. It was not tender and was freely movable. Under local anesthesia the mass was excised but unfortunately its depth was misjudged by the surgeon, and during the procedure it was incised. At this point a thread-like structure resembling a worm was noted and in order to make certain that all of the parasite was removed the incision was extended, care being taken not to leave any portion of the hair-like structure behind. The lesion healed uneventfully and there has been no recurrence.

The specimen consisted of several masses of fibrous tissue containing a considerable amount of fat, two worm-like structures approximately 1.25 and 1.0 cm in length respectively, and several smaller fragments of a worm or worms. On closer inspection it appeared that only one worm was present and that it had been severed in several places during surgery (Figure 1). One of two microscope slide preparations submitted was found to contain cross sections of the worm (Figure 2). The gross appearance and microscopic configuration of the worm specimens were compatible with the sparganum of *Spirometra*.

As a part of the study of the epidemiology of *Echinococcus granulosus* in Mississippi, a number of wild animals have been trapped and autopsied. In 1957 this series included seven bobcats (*Lynx rufus*), two of which were found to harbor adult pseudophyllidean tapeworms. These specimens were initially studied in our laboratory and tentatively identified as *Spirometra mansonoides*. Later they were sent, along with the surgical specimens from Case 2, to Dr. J. F. Mueller who confirmed the species identification of the adult worm.

COMMENT

As it is possible to arrive at a species diagnosis only upon examination of the adult worm, and there was no opportunity to feed these spargana to an appropriate definitive host, it cannot be stated that the larval worms were *Spirometra mansonoides*. Inasmuch as the adult of this species is known to occur in bobcats in the same geographic area, there is presumptive evidence that the observed spargana were of the same species.

SUMMARY

Two cases of human sparganosis are reported. Both occurred in white women, and both are believed to have originated in Mississippi. Neither of the patients had ever left the United States.

Two of seven bobcats (*Lynx rufus*) captured in Mississippi during the summer months of 1957 were found to harbor adult *Spirometra mansonoides*, and it is suggested that the observed spargana may be of the same species.

REFERENCES

1. Mueller, J. F., 1935. A *Diphyllobothrium* from cats and dogs in the Syracuse region. *J. Parasitol.*, **21:** 114–121.
2. Mueller, J. F., 1951. Sparganа from the Florida alligator, *J. Parasitol.*, **37:** 317–318.
3. Faust, E. C., Campbell, H. E., and Kellogg, C. R., 1929. Morphological and biological studies on the species of *Diphyllobothrium* in China. *Am. J. Hyg.*, **9:** 560–583.
4. Mueller, J. F., 1937. A repartition of the genus *Diphyllobothrium*. *J. Parasitol.*, **23:** 308–310.
5. McIntosh, A., 1935. New host records for *Diphyllobothrium mansonoides* Mueller. *J. Parasitol.*, **23:** 313–315.
6. Mueller, J. F., 1938. The life history of *Diphyllobothrium mansonoides* Mueller, 1935, and some considerations with regard to sparganosis in the United States. *Am. J. Trop. Med.*, **18:** 41–66.
7. Mueller, J. F., and Coulston, F., 1941. Experimental human infection with the sparganum larva of *Spirometra mansonoides* (Mueller, 1935). *Am. J. Trop. Med.*, **24:** 399–425.
8. Stiles, C. W., 1908. The occurrence of a proliferating cestode larva (*Sparganum proliferum*) in man in Florida. *Hyg. Lab. Bull.*, No. **40:** 7–18.
9. Moore, J. T., 1915. *Sparganum mansoni*. First reported American case. *Am. J. Trop. Dis. & Prev. Med.* **2:** 518–525.
10. Botero, D., and Gomez, J. J., 1958. The first case of sparganosis in Colombia. *Am. J. Trop. Med. & Hyg.*, **7:** 597–599.

ON THE VIRULENCE OF THE GEOGRAPHIC STRAINS OF *SCHISTOSOMA JAPONICUM**

S. Y. LI HSÜ AND H. F. HSÜ

Department of Hygiene and Preventive Medicine, College of Medicine, State University of Iowa, Iowa City, Iowa

During the time when we visited different endemic areas of schistosomiasis japonica in the Far East, we often noticed the different degrees of damage caused by this disease geographically. In certain villages, the disease had gradually decimated the inhabitants; in a few, annihilation had occurred; in others, it had caused a moderate degree of damage; and in still others, the inhabitants seemed to tolerate the disease well. We fully realize that schistosomiasis japonica is not the only factor which caused such an apparent difference in the health of the inhabitants of the endemic areas. However, it was suggested to us that different geographic strains of *Schistosoma japonicum* may differ in virulence, especially after the existence of four biologically distinct geographic strains of the schistosome species was established.[1] We have, therefore, investigated whether differences in virulence can be demonstrated in laboratory animals infected with the four geographic strains of this parasite.

MATERIALS AND METHODS

Four geographic strains of *S. japonicum* were used: the Chinese, Formosan, Japanese, and Philippine. The cercariae of the Chinese strain used were obtained from *Oncomelania hupensis* originating in Kashing, Chekiang, China (Vogel's strain); the Formosan, from *O. formosana* obtained from Changhua, Taiwan, China; the Japanese, from *O. nosophora* obtained from Kofu, Japan; and the Philippine, from *O. quadrasi* obtained from Leyte, the Philippines. Albino mice of 17 to 19 grams were used as the experimental animals, and were infected by the cutaneous route. The sex of cercariae shed from an infected snail was determined by a previous inoculation to a mouse. Only those snails which were shedding one sex of cercariae were used. Each mouse was exposed to a definite number of cercariae with the sexes equally divided, *i.e.*, 100 cercariae means 50 males and 50 females. Survival of the mice was recorded in days after inoculation as well as days after eggs had been found in the stools.

Whenever abbreviations are used in the text, C means Chinese; F, Formosan; J, Japanese; and P, Philippine.

RESULTS

1. *Survival of mice infected with different numbers of cercariae of the F strain*

To determine the number of cercariae to be used for the virulence tests, four batches of mice were infected with 12, 24, 50, and 100 cercariae respectively. Each batch consisted of 36 mice. An additional batch of 36 mice served as control animals. In the control series, two mice died, one on the 103rd day and the second on the 312th day of the experiment. The means of the survival times of the above-mentioned four series of infected mice are given in Table 1.

In general, the survival time of infected mice became shorter as the number of inoculated cercariae increased. The results obtained in the 100-cercariae series deserve special consideration. As in the case of the other series with lighter infection, no mice died in the prepatent period in this series. The mean and standard deviation of the survival times of the animals in this series were very short—only 8.19 ± 4.59 days after the eggs were found in the stools. Until the end of the experiment (68 days), no mice died in the concurrent control series. Accordingly, among these four intensities of infections, the dose of 100 cercariae seemed to be the most suitable for study of the virulence of *S. japonicum*.

Previously we had infected 19 mice with 200 male cercariae of the J strain. Among them, 11 mice died within 21 days after the infection. This result indicates that if mice were infected

* This investigation was supported by Research Grant E-939, National Institute of Allergy and Infectious Diseases of the National Institutes of Health, United States Public Health Service, and by a research grant from Abbott Laboratories, North Chicago, Illinois.

TABLE 1

Means and standard deviations of survival times of mice infected with 12, 24, 50, and 100 cercariae of the F *strain of* S. japonicum. *The number of mice was 36 in each series*

Number of cercariae	Survival time in days					
	After infection			After eggs found in the stools		
	Mean	S.D.	Range	Mean	S.D.	Range
12	101.00	72.73	45–394	56.43	72.13	3–353
24	60.83	9.49	43–96	17.69	8.57	1–46
50	53.56	9.07	40–79	10.86	8.05	0*–35
100	50.22	5.91	41–68	8.19	4.59	2–23

* "0" means that at the time of death eggs had not been found in the stool.

TABLE 2

Means and standard deviations of the survival times of mice infected with 100 cercariae of the C, F, J, *and* P *strains of* S. japonicum. *The number of mice was 36 in each series*

Strain	Survival time in days					
	After infection			After eggs found in the stools		
	Mean	S.D.	Range	Mean	S.D.	Range
C	47.89	3.72	41–61	6.94	3.61	0*–19
F	50.22	5.91	41–68	8.19	4.59	2–23
J	38.78	1.63	37–41	4.50	1.61	0*–7
P	40.61	2.17	35–47	5.17	1.99	0*–13

* "0" means that at the time of death eggs had not been found in the stool.

with 200 cercariae with sexes equally divided, some might die before the deposition of eggs in the visceral organs. In the present study, we intend to compare the survival time of infected mice after the eggs of the worms have been deposited in the visceral organs. Therefore, an infecting dose of 100 cercariae was chosen.

2. *Survival time of mice infected with 100 cercariae of one of the four geographic strains*

The mean of the survival times of the 36 mice infected with 100 cercariae of the F strain has been given above. Three other batches of mice were used for the infection of the C, J, and P strains. Each batch also consisted of 36 mice and each mouse was infected with 100 cercariae. An additional batch of 36 mice served as controls.

No control mice died throughout the 68 days of experiments. The means and standard deviations of the survival times of mice infected with 100 cercariae of the four strains are given in Table 2.

Mice infected with the J strain had the shortest survival time and those with the F strain the longest; mice infected with the P and C strains lay between those of the J and F strains. The mean survival time of mice infected with the P strain was shorter than that of the C strain. The standard error test of significance of the differences between the means* shows that, for the means of the survival times of mice after infection, the differences were significant at the 5% level between any two of the four strains. The difference between the C and F strains, however, was apparently borderline (d = 2.00). In this one case, we would be inclined to say that, while the result was suggestive, it was not quite firmly established. All of the other results were sufficiently solid. The same test shows that, with the means of the survival times of mice after eggs were found in the stools, there were neither significant differences between the means of the J and P strains nor between the means of the F and C strains, although there were significant differences at the 5% level between the means of the following strains: J vs. C, J vs. F, P vs. C, and P vs. F. It is interesting to note here that in the results obtained from the tests for the after-infection differences in means,

* Before the standard error test of significance of the differences between the means was performed, Bartlett's test for the homogeneity of variances had been done, showing that the variances were heterogeneous. It is, therefore, not legitimate in this case to use the F test for the analysis of the variance. Since all the four means resulted from large samples, it seems appropriate to use the standard error test to make pairwise comparisons of the means. Dr. Lillian R. Elveback, Head of the Biostatistics Division, Department of the Tropical Medicine and Public Health, Tulane University, kindly performed a non-parametric test on the present data. She compared the four survival time distributions (after infection) by the chi-square test, which is free of any assumptions on the standard deviations, and found a very clear result showing that the evidence was sufficient to justify the conclusion that the distributions of survival times were not the same for the four strains. We wish to express our sincere appreciation for her interest.

TABLE 3

Proportion of mice showing intestinal hemorrhages during the course of infection and at autopsy. The number of mice was 36 in each series, and each mouse was infected with 100 cercariae of the C, F, J, *and* P *strains*

Strain	Percentage of mice showing intestinal hemorrhages		
	(a) Blood in the stools during the course of infection	(b) Blood in the intestines during the autopsy	(a) and/or (b)
C	22.22	11.11	22.22
F	46.88	37.50	68.75
J	66.67	16.67	66.67
P	33.33	13.89	35.11

the relative deviates were also smallest for the C vs. F and J vs. P, and, as mentioned above, the C-vs.-F result was even doubtful.

As the prepatent periods of the four strains of *S. japonicum* in mice infected with 100 cercariae have been found to be different,[2] that portion of the survival time of infected mice between the appearance of eggs in the stools and the death of the host should be chosen as the basis for comparison of the virulence of the strains. It seems justified to conclude that the J and P strains are more virulent than the C and F strains, but that the differences in virulence between the C and F strains and between the J and P strains are not significant.

3. *Intestinal hemorrhages in infected mice*

During the course of infection, bloody stools were sometimes seen in infected mice. During autopsies, hemorrhages in the intestines, principally in the cecum and colon, were also seen. In certain instances, the death of the animals may be attributed to the great loss of blood in the intestines. It is, therefore, important to investigate whether the frequencies of the hemorrhagic findings in mice infected with the four strains of *S. japonicum* have a definite correlation with the virulence of the strains.

Throughout the course of the experiments, we recorded macroscopically bloody stools, if any. Whenever the autopsy of an infected mouse was performed, hemorrhages in the intestines were also noted. In nearly all cases, hemorrhages, if present, were heavy, with cecum or colon or both filled with blood. Only in five mice (one infected with the F strain, three with the J strain, and one with the P strain), was a moderate degree of hemorrhage observed in the distal part of the ileum. The proportion of mice showing hemorrhages is given in Table 3. The data show that hemorrhages were most frequent in mice infected with the F strain. As the F strain has been shown to be the strain of the least virulence among the four, it indicates that intestinal hemorrhages in infected mice should not be regarded as an important factor in determining the virulence of strains of *S. japonicum*.

DISCUSSION

The fact that the virulence of the four geographic strains of *S. japonicum* was different in infected mice seems to be well established by the results of the present study. It is interesting to note that the more virulent the strain is, the shorter the prepatent period of the strain. That the more virulent strain usually shows a shorter incubation or prepatent period is a general phenomenon of infectious microorganisms.

The possibility that the early death of mice may be due to larger worms or larger numbers of worms crowding the portal or mesenteric veins should first be investigated. In the present series of experiments of mice inoculated with 50 male and 50 female cercariae of either of the four strains of *S. japonicum*, infarcts were observed neither in the liver, spleen, stomach, nor intestine during necropsies. Results of experiments in this laboratory (unpublished data) indicate that mice infected with 100 male cercariae of either the F or J strain usually do not die within 100 days after infection. It thus shows not only that the death of mice in the present series of experiments was not due to the crowding effects of the worms in the portal or mesenteric veins but also that the main cause of the deaths was not the toxic metabolic products of the worms.

The nature of the virulence of *S. japonicum* is a problem worthy of further investigation. In their studies on the histopathology of experimental schistosomiasis, Meleney *et al.*[3] ascertained that the principal pathological lesions are caused by eggs in the infested organs, and that the individual egg of *S. japonicum* is more toxic to the tissues of the host than is that of *S. mansoni*. While we are not certain whether the secretions of the eggs of the different strains

of *S. japonicum* differ qualitatively in their toxicity, results of studies in progress do suggest that the quantities of eggs produced by this schistosome species may be an important factor in the virulence of its various geographic strains

SUMMARY

The virulence of the four geographic strains of *S. japonicum* was found to be different in mice infected with 100 cercariae. Judging from the host's survival times after inoculation and after the appearance of eggs in the feces, the Japanese and Philippine strains of this schistosome species were shown to be more virulent than the Chinese and Formosan strains.

REFERENCES

1. Hsü, H. F., and Hsü, S. Y. Li, 1959. Characteristics of geographic strains of *Schistosoma japonicum* in the final hosts. *Proc. 6th Internat. Congr. Trop. Med. Malaria*, Lisbon, September, 1958 (in press).
2. Hsü, H. F., Hsü, S. Y. Li, and Tsai, C. T., 1960. Further studies on the prepatent periods of four geographic strains of *Schistosoma japonicum*. *Libre Homenaje al Dr. Eduardo y Caballero* (in press).
3. Meleney, H. E., Sandground, J. H., Moore, D. V., Most, H., and Carney, B. H., 1953. The histopathology of experimental schistosomiasis. II. Bisexual infections with *S. mansoni*, *S. japonicum*, and *S. haematobium*. *Am. J. Trop. Med. & Hyg.*, **2**: 883–913.

THE EFFECTS OF LOW CONCENTRATIONS OF SODIUM PENTACHLOROPHENATE ON THE FECUNDITY AND EGG VIABILITY OF *AUSTRALORBIS GLABRATUS*

LOUIS OLIVIER AND WILLARD T. HASKINS*

U. S. Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, National Institute of Allergy and Infectious Diseases†, Bethesda, Maryland

Sodium pentachlorophenate (NaPCP) is known from laboratory[1, 2] and field[3-9] experience to be highly toxic to the aquatic snails which are the vectors of schistosomiasis. The chemical has been tested in a variety of concentrations but perhaps the most commonly used procedure in the field is to apply 10 parts per million (ppm) for 8 hours in flowing water and to use lower concentrations in still water.

Very low concentrations of the chemical have had little trial against adult snails. In the laboratory Halawani *et al.*[10] killed 1 of 4 snails at 0.5 ppm and 4 of 4 snails at 1.0 ppm in 96 hours. Newton and Haskins[11] exposed adult *Australorbis glabratus* of four strains to concentrations of the chemical ranging from 0.25 to 1.5 ppm for 24 hours at 27°C. They found differences in susceptibility and in LD_{50} values which ranged from 0.68 to 1.06 ppm. When two strains were exposed continuously to 0.5 ppm all of the snails of one strain were dead in 80 hours while 67% of the other strain lived 7 days. Nolan *et al.*[1] found NaPCP at 1 ppm to be essentially ineffective when used for 24 hours. Klock *et al.*[8] used concentrations of NaPCP ranging down to 0.5 ppm and found that at the latter concentration some of the snails survived 11 days.

There appear to be no published reports of the use of NaPCP at very low concentrations against adult snails in the field.

Few reports have dealt with the destruction of snail eggs by NaPCP. Berry *et al.*[4] noted that in one field experiment the chemical, when used at about 9.5 ppm for 6 hours, killed eggs of *A. glabratus*. Dobrovolny and Barbosa[6] found all *A. glabratus* and *Tropicorbis centimetralis* eggs dead after 10 ppm were used for 10 hours and, generally, when all snails were killed by the chemical all the eggs were also killed. Halawani *et al.*[10] found that 10 ppm killed the eggs of the schistosome vectors in Egypt and Kuntz[7] observed that 2 and 5 ppm for 24 hours killed about 75 and 90% respectively of the eggs of *Biomphalaria*. Parent and Lietar[12] found that NaPCP at 10 ppm for 6 hours did not kill the eggs of *Biomphalaria* and *Physopsis*. Newton and Nolan (unpublished) exposed numerous eggs of *A. glabratus*, having embryos in all stages of development, to 5 and 10 ppm for 6 to 24 hours. Three percent of the eggs hatched, the greatest proportion of survivors was in the groups treated with 10 ppm for 6 hours, and there was evidence that the older embryos were less readily killed than the young embryos. Chaia *et al.*[13] found that all *A. glabratus* eggs were killed by 10, 20, and 40 ppm in 6 to 9 hours. Klock *et al.*[8] using concentrations of 3 to 200 ppm found that the toxicity was about the same for ova as for adult *A. glabratus* but that it was necessary to expose ova longer than the adults in order to kill all of them.

The experiments discussed here were designed to test the effect of very low concentrations of NaPCP on the fecundity of *Australorbis glabratus* and on the viability of the eggs produced while the snails are exposed to these low concentrations of the chemical. The concentrations chosen for study, 0.05 and 0.1 ppm, were well below those known to have been used before. Nevertheless, striking effects on both fecundity and egg viability were observed.

MATERIALS AND METHODS

The snails used were from two laboratory strains of *A. glabratus*. One, designated "M", was the unpigmented strain XXIX-M of Newton.[14] The other, also an unpigmented strain, was derived from Venezuelan stock and is here designated as "Vm." All snails used were young specimens raised in the laboratory under closely similar conditions. The snails used in any given experiment were of approximately the same age

* With the technical assistance of Mr. Ned M. Etherington

† Laboratory of Parasitic Diseases

and size and had recently reached sexual maturity. They were maintained throughout the course of the experiments in cylindrical battery jars containing 2.5 liters of water. All jars were given identical care throughout the course of the experiment save for the introduction of the chemical into the test jars. The snails were fed fresh Romaine lettuce which was changed daily. The laboratory temperature ranged from 26 to 28°C and the water temperature ranged from 25 to 27°C.

Fecundity was studied by counting all the eggs laid in each jar every day. The eggs were collected once each day and were not returned to the jars. Collection and observation of the eggs was greatly facilitated after the discovery that when thin (thickness: 0.0015 inch) polyethylene film (from bags used for home food storage) was floated on the water of the jars a large proportion of the eggs were laid on the plastic sheet instead of on the glass side of the jar which is the usual site. In the experiments to be reported here 79.2% of the 171,956 eggs produced were laid on the plastic. The film could be removed to another container to await study without disturbing the eggs. Moreover, the eggs could be examined under the microscope without the necessity of scraping them from the substrate since the plastic is transparent. In the experiments reported here the plastic sheets were removed daily and small discs of plastic, each containing a single egg clutch, were cut from the sheets with a sharp brass cork borer. These discs were put into numbered dishes and held for examination and egg counts later in the day. A disc can be handled with fine forceps without the need to touch the attached egg clutch. Eggs laid on the sides of the jars were counted with the aid of a hand lens and then removed after they were scraped free with a razor blade.

The plastic sheets were returned to the jars in a random fashion in order to minimize possible variables in suitability of the plastic sheets for egg deposition. However, a sheet that had been in a jar with chemical was never put into a control jar.

New plastic sheets do not appear to affect the health of adult snails but the snails do not use them for egg deposition. New sheets may be used after washing once in hot water and detergent and then soaking for 2 weeks in tap water or until water wets the surface well. The plastic had no effect on the NaPCP in the concentrations used.

Egg viability was studied by examining daily all eggs laid on the plastic with a dissecting microscope and recording the number of dead embryos found. Thus at the time of examination the eggs had been exposed to the chemical for up to 24 hours.

A stock solution of sodium pentachlorophenate containing 0.1 mg of pentachlorophenol per ml was prepared by dissolving 10.0 mg of pure pentachlorophenol (Eastman Organic Chemicals) in 5 ml of saturated sodium bicarbonate solution and diluting to 100 ml with distilled water. This solution is stable for several weeks in the refrigerator. The volume of the water in the jars containing the snails was adjusted to 2.5 liters and the fluid level marked by a line on the outer surface of the glass. For the experiments requiring 0.05 ppm concentration of the molluscicide the stock solution was diluted to contain 0.025 mg per ml. Five ml of this was pipetted into each jar to be treated. After each addition the contents of the jar were stirred to insure even distribution of the chemical. A similar procedure was followed for the experiments requiring 0.1 ppm solutions except that 2.5 ml of the undiluted stock solution was added to each jar. Evaporation of water from the jars was not a serious problem for the 7 or 8 days between the changes but, when necessary, water was added to maintain the proper level.

An attempt was made to follow the NaPCP concentration analytically from day to day during exposure but it was soon found that this was not feasible for two reasons. At these low concentrations it would have been necessary to remove at least two, and preferably three, 50-ml samples per day for the analyses. This would have drastically reduced the volume in the jars in a week's time. Also, preliminary analyses on the control jars without chemical in which the snails had been living for several days gave widely varying blank readings from jar to jar. This would preclude any reliable results from determinations on the NaPCP solutions from the treated jars. This variable blank appeared to be related in part to the degree of cloudiness of the water in the jars which in turn is related to the amount of molds, bacteria, and protozoa present. High blank readings were obtained from jars in which the water was obviously cloudy.

TABLE 1

The fecundity of Australorbis glabratus *exposed to sodium pentachlorophenate, and the viability of eggs recovered during exposure*

Experiment	NaPCP Conc. (ppm)	Snail strain			Pre-test period (days)								Test period (days)								Recovery period (days)							
					−8	−7	−6	−5	−4	−3	−2	−1	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
1	0.05	M	E/s/d§	C†	0	.4	1.7	2.0	3.2	5.0	2.2	4.1	6.3	6.9	4.8	6.2	9.9	6.1	9.3	5.7	11.0	9.8	8.1	5.8	8.3	13.1	13.4	
			E/s/d	T‡	.6	.4	2.3	2.1	4.3	4.6	4.2	5.5	4.7	9.1	8.8	9.0	10.7	9.3	10.9	10.3	13.5	14.6	7.0	10.5	11.9	14.5	16.8	
			V.‖	C	0	0	0	0	0	0	0	0	0	0	0	0	3.2	0	0	2.0	.5	.2	12.5	0	1.3	1.0	.3	
			V.	T	0	0	0	0	0	0	0	0	100.0	93.3	74.7	54.1	9.1	54.0	9.8	35.0	.5	1.0	1.6	1.4	1.6	.7	.5	
2	0.05	Vm	E/s/d	C		3.4	8.6	9.3	9.5	10.6	9.5	16.4	15.1	17.5	16.4	17.1	20.7	16.8	14.1		22.3	20.9	18.4	14.6	17.4	12.2	12.9	12.9
			E/s/d	T		3.6	8.3	8.8	9.0	9.3	10.8	11.7	3.5	4.7	9.3	8.4	7.5	7.8	6.7		12.5	10.2	10.3	10.8	13.8	10.3	6.3	12.6
			V.	C		1.3	0	.6	.2	1.2	0	0	0	.7	1.2	.1	1.2	.5	.9		0*	.4	.5	.5	.1	.4	1.2	0
			V.	T		.6	0	0	.1	2.2	.6	.3	0	74.3	93.7	70.9	88.4	94.7	70.8		0*	20.7	19.0	16.4	2.0	23.1	1.7	1.2
3	0.1	M	E/s/d	C		12.6	12.0	7.0	7.9	9.4	15.2	15.7	10.9	13.3	9.9	9.6	6.6	14.2	15.3		14.3	15.8	13.7	17.3	17.6	23.9	22.7	
			E/s/d	T		11.5	11.5	8.1	8.6	11.0	12.9	14.6	.5	1.0	1.8	1.3	.2	1.1	.9		6.0	3.9	6.4	5.6	6.6	9.1	9.6	
			V.	C		1.1	1.2	2.0	1.8	1.5	.5	.5	1.8	.4	7.5	.3	0	.3	.5		.1	.4	0	.2	1.4	2.0	.9	
			V.	T		.2	.8	11.7	.1	1.7	1.2	.3	0	34.7	80.3	40.2	16.7	36.8	84.5		10.0	1.4	2.6	7.0	4.7	5.4	7.8	
4	0.1	Vm	E/s/d	C	17.6	16.0	16.1	9.2	15.7	13.7	9.1	13.5	21.0	15.3	20.0	22.7	15.6	18.0	22.2		23.0	17.9	20.6	21.3	15.7	19.2	17.8	
			E/s/d	T	17.2	15.1	13.1	16.3	15.5	8.7	10.2	12.0	.6	.3	.2	1.3	1.1	1.2	1.6		4.6	7.3	8.5	11.7	5.3	12.3	11.1	
			V.	C	0	7.1	9.4	12.6	.1	13.2	3.2	.4	.4	.7	.1	.8	.5	.2	.3		1.0	.4	1.8	1.2	.2	.6	.3	
			V.	T	0	2.6	5.2	2.9	1.9	8.9	0	1.1	100.0	100.0	94.4	100.0	53.8	50.0	37.1		1.8	4.5	16.4	38.8	6.1	31.3	19.7	

* Relatively few eggs were laid on the plastic on this date due to the use of unsuitable plastic.

† Control group.

‡ Test group.

§ Egg production expressed as eggs per snail per day.

‖ Viability of the eggs expressed as the percentage of eggs laid on the plastic which were dead.

EXPERIMENTS

Four experiments were conducted using NaPCP concentrations of either 0.05 or 0.1 ppm. These concentrations were chosen because previous experiments using 24-hour exposures had indicated they were probably at, or below, the limit of toxicity of the chemical for the adult snails. From 93 to 100 snails of a single strain were used as test animals and a like number were used as controls in each experiment. The number of snails per jar at the start of the experiment ranged from 8 to 10.

In each experiment there was a pre-test period of 7 or 8 days when all the snails were kept in untreated water. During this time the daily production of eggs in each jar was recorded as eggs per snail per day (e/s/d). The eggs laid on the plastic were all examined to establish their viability pattern. Egg production and egg viability in the test and control jars were essentially similar during the pre-test period in all experiments. In experiment 4 the water of all jars was changed on the 7th day of the pre-test period since falling egg production was presumed to be due to the development of unfavorable conditions in the jars.

At the end of the pre-test period the desired amount of the chemical was added to the 10 test jars. Daily egg counts and examinations for egg viability were continued for the test period which lasted either 7 or 8 days. During this period no additional chemical was added and the water was not changed.

At the end of the test period the water of all jars was changed and replaced with untreated water. The experiment was then continued for 7 or 8 more days in order to observe possible recovery of egg production and egg viability. In Experiment 1 the water of all jars was also changed on the 5th day of the recovery period.

Dead snails were removed daily when the eggs were collected. In Experiment 1 there were 100 snails in each group. Four test and 8 control snails died during the pre-test period and were replaced when found. In the remainder of the experiment 9 test and 15 control snails died and were not replaced. In Experiment 2 there were 100 snails in each group. In the pre-test period 1 test and 2 control snails died and were replaced when found. In the remainder of the experiment 5 test and 3 control snails died but were not replaced. At the start of Experiment 3 there were 94 test and 93 control snails. Six test and 5 control snails died during the pre-test period. At the start of the test period the number of snails was raised to 100 in each group. In the remainder of the experiment 23 test and 10 control snails died and were not replaced. At the start of

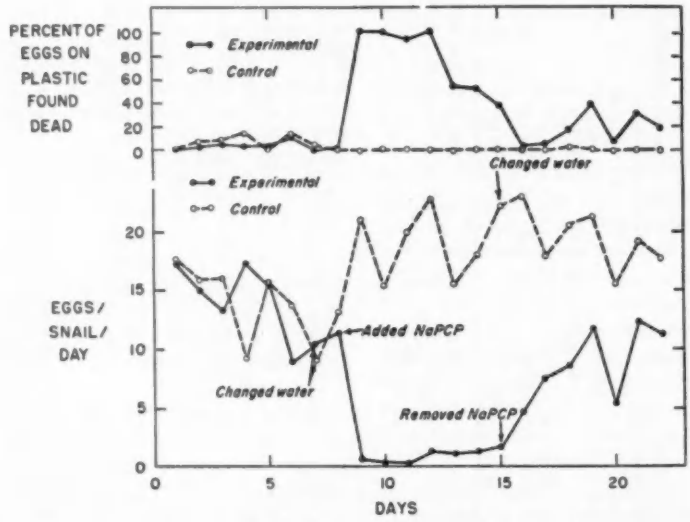


FIG. 1. Egg production and egg viability of *Australorbis glabratus* (strain Vm) exposed to 0.1 ppm of sodium pentachlorophenate. (Experiment 4).

Experiment 4 there were 99 test and 98 control snails. In the pre-test period 4 test and 1 control snail died. At the start of the test period the number of snails in both groups was raised to 100. In the remainder of the experiment 26 test and 2 control snails died and were not replaced. When dead snails were replaced during or at the end of the pre-test period this was done after the daily egg count had been made. Such replacement of snails increased egg counts but did not affect the pattern of egg production. The number of snails alive when the eggs were counted was used for purposes of calculating egg production rates. This number, rather than the number of snails at the start of the day, was preferred.

The essential data from the 4 experiments are summarized in Table 1 and the data from Experiment 4 are plotted in Figure 1.

DISCUSSION

Obviously the NaPCP, as used in these 4 experiments, had important effects on the fecundity of *A. glabratus* and the viability of the eggs produced. At 0.05 ppm the snails in one experiment continued to produce eggs normally but in the other production of eggs was greatly reduced. The effect of 0.1 ppm on the fecundity of the snails was even more striking.

The eggs laid during the time the snails were exposed to either 0.05 or 0.1 ppm of the chemical had greatly reduced viability. A gradual improvement in egg viability during the test period in two experiments (1 and 4) may have been due to a decline in concentration of the chemical in the water during the test period since none was added and the level could not be measured.

The effects of the chemical, as used, on egg production and on egg viability are striking when considered separately but they are even more impressive when the additive effect of the two actions is considered. For example, in Experiment 3 the recovery of *viable* eggs on the day the test period started was 14.5 e/s/d (14.6 less the eggs dead on that day). During the subsequent test period the recovery of *viable* eggs varied from 0.12 to 0.78 e/s/d (eggs recovered less dead eggs). In Experiment 4, 0.1 ppm of the chemical reduced the production of *viable* eggs from 11.9 e/s/d before the addition of chemical to from 0 to 1.03 e/s/d during the test period. It should also be noted in this connection that the eggs were checked for viability on the day of collection and then discarded. For this reason evidence of damage to the eggs which might have appeared later was not taken into account. Moreover, when destruction of the adult snails by 0.1 ppm of the chemical is considered, the over-all effect becomes even more striking. Indeed, if the calculations are based on 100 snails, the *viable* egg recovery was reduced to 0.11 egg per original snail and 0.81 egg per original snail on the last day of the test period in Experiments 3 and 4 referred to above. These are, respectively, 132-fold and 15-fold reductions from the pre-test levels.

Removal of the chemical was followed by greater egg recovery and increased egg viability. In the 3 experiments in which egg production was depressed the production of eggs increased slowly but steadily after removal of the chemical and large gains in egg productivity were made in 2 of the 3 experiments within one week. More rapid recovery was made in egg viability but an appreciable number of dead snails were found during the recovery period in 3 of the 4 experiments.

The precise manner in which the NaPCP acts to inhibit egg production of the snails is not known but since this chemical is a powerful uncoupler of oxidative phosphorylation[15] it is reasonable to assume that its action on egg production derives from this fact.

Destruction of the eggs probably results from this same cause. However, it is probable that the chemical reaches the eggs in two ways. The data on egg viability during the recovery period suggest that the eggs may be affected before they are laid since during the recovery period there were more dead snails in the test groups than in the control groups. But it is probable that the eggs were also affected by contact with the NaPCP after they were laid. Immediately following extrusion of the egg mass by the snail the material enveloping the eggs imbibes water. By this means the chemical in the water may presumably be drawn into close contact with the uncleaved eggs. Unpublished experiments in this laboratory have demonstrated that freshly laid eggs are highly susceptible to NaPCP.

Eventual recovery of a high level of egg production and egg viability after exposure of the snails to the chemical demonstrate that the NaPCP did not destroy the capacity of the snails to produce eggs. The relatively slow and gradual

resumption of egg production and the persistence of a small but significant proportion of dead eggs in the collections suggest that the chemical may have accumulated in the snails during their exposure and so continued to affect egg production and viability for a time after the environment ceased to be toxic. Weinbach and Nolan[16] have shown that *A. glabratus* can accumulate NaPCP from water containing 2 ppm of the chemical. It is not known that they can take up the chemical from more dilute solutions but there seems no reason to believe this would not occur.

The experiments tend to show that 0.05 ppm is at the lower useful limit of effectiveness on egg production and egg viability of the snail strains used. Doubling the concentration resulted in much more striking effects on egg production and viability. The death losses among the adult snails also indicate that the concentrations tested are at the limit of effectiveness against the adult snails.

Although available evidence is inadequate to establish the point, it is probable that the differences observed in the responses of the two snail strains were in part due to inherited factors. Newton and Haskin's work[11] and unpublished experiments in this laboratory have given evidence to support this hypothesis.

The results reported here give quantitative evidence for the efficacy of very low concentrations of NaPCP in reducing egg productivity of *A. glabratus* in the laboratory. These results encourage the hope that similar concentrations of the chemical may prove to be useful and practical in the field. Highly diluted $CuSO_4$ has already been used in the field. Sharaf El Din and El Nagar[17, 18] undertook to control the schistosome vectors in the Gezira irrigated area of the Sudan by continuously introducing $CuSO_4$ into irrigation canals at a level calculated to be 0.125 ppm. They reported successful control of the vector snails.

All previous field experiments with NaPCP have been designed primarily to kill the adult snails, and the measures of the chemical's efficacy have usually been in terms of adult snail mortality after a short-term treatment with the chemical. Nevertheless, egg destruction and the inhibition of egg production might be effective for schistosome vector control in some situations. Total destruction of the vector snail population is not required to significantly reduce transmission of schistosomiasis and, indeed, eradication of the snails from any endemic area can hardly be hoped for within the forseeable future. Interference with the reproductive capacity of the snails by the use of very low concentrations of the chemical, even if the adult snails are not destroyed, might reduce or interrupt transmission of schistosomiasis at relatively low cost. The vector snails in some endemic areas are known to have pronounced seasonal population cycles[19-22] and it may be that in these and other endemic situations egg laying may be confined to a relatively short season of the year. Suppression of egg production in these periods might give substantial vector control for a whole season or even for a year. Another possibility is that very low concentrations of the chemical might be used on a maintenance basis after a larger concentration is used to kill most of the adult snails. The continued use of the chemical at very low concentration under these conditions might prevent the re-establishment of the snail population. Only field experiments after suitable study of the snail life cycles in endemic areas can test these possibilities and reveal whether prolonged use of very low concentrations of NaPCP would be effective and cost less than repeated use of higher concentrations for shorter periods.

SUMMARY

Australorbis glabratus adults were placed in either 0.05 or 0.1 ppm of sodium pentachlorophenate for 7 or 8 days and the effects of this treatment on egg production and on viability of the eggs laid were determined. In general the treatment drastically reduced egg production and a very large proportion of all eggs recovered from the aquaria were dead. Removal of the chemical resulted in improved egg production and egg viability. The possible significance of these results for field control of the snail vectors of schistosomiasis is discussed.

REFERENCES

1. Nolan, M. O., Bond, H. W., and Mann, E. R., 1953. Results of laboratory screening tests of chemical compounds for molluscicidal activity. I. Phenols and related compounds. *Am. J. Trop. Med. & Hyg.*, **2**: 716–752.
2. Perlowagora-Szumlewicz, A., and de Oliveira Dias, C., 1955. Experiências sôbre a rapidez da ação moluscocida do sulfato de cobre e pentaclorophenato de sodio êm relação ao contacto e à concentração. *Rev. Brasil. Med.*, **12**: 295–303.
3. Nolan, M. O., and Berry, E. G., 1949. Pre-

liminary field trials with laboratory-tested molluscacides. *Pub. Health Rep.*, **64:** 942–949.

4. Berry, E. G., Nolan, M. O., and Oliver González, J., 1950. Field tests of molluscacides against *Australorbis glabratus* in endemic areas of schistosomiasis in Puerto Rico. *Pub. Health Rep.*, **65:** 939–950.
5. Dobrovolny, C. G., and Barbosa, F. S., 1953. Field trials of sodium pentachlorophenate as a mulluscacide in flowing waters in Brazil. *Pubs. Avulsas Inst. Aggeu Magalhães, Recife*, **2:** 121–158.
6. Vaughn, C. M., Olivier, L., Hendricks, J. R., and Mackie, T. T., 1954. Molluscicide operations in an endemic area of schistosomiasis in the Dominican Republic. *Am. J. Trop. Med. & Hyg.*, **3:** 518–528.
7. Kuntz, R. E., 1956. Evaluation of sodium-pentachlorophenate as a molluscicide in Egypt. *Am. J. Trop. Med. & Hyg.*, **5:** 274–285.
8. Klock, J. W., Gerhardt, C. E., Ildefonso, V., and Mateo Serrano, J., 1957. Characteristics of sodium pentachlorophenate used against *Australorbis glabratus* in Puerto Rico. *Bull. World Health Organ.*, **16:** 1189–1201.
9. Wright, W. H., Dobrovolny, C. G., and Berry, E. G., 1958. Field trials of various molluscicides (chiefly sodium pentachlorophenate) for the control of aquatic intermediate hosts of human bilharziasis. *Bull. World Health Organ.*, **18:** 963–974.
10. Halawani, A., Latif, N., and Taha, A., 1951. On pentabromophenol and pentachlorophenol as molluscacides in the prevention of bilharziasis. *J. Roy. Egyptian M. A.*, **34:** 163–170.
11. Newton, W. L., and Haskins, W. T., 1953. A comparison of the dosage-mortality responses of some strains of *Australorbis glabratus* to sodium pentachlorophenate. *Am. J. Trop. Med. & Hyg.*, **2:** 708–715.
12. Parent, M., and Lietar, J., 1955. Contribution à l'étude de la biologie des mollusques à Jadotville. *Ann. Soc. Belge Med. Trop.*, **35:** 59–67.
13. Chaia, G., Paulini, E., and Queiroz, A. B., 1956. Ação do pentaclorofenato de sodio e do sulfato de cobre sôbre os ovos de *Australorbis glabratus* (Say). *Rev. Brasil. Malariol.*, **8:** 605–612.
14. Newton, W. L., 1953. The inheritance of susceptibility to infection with *Schistosoma mansoni* in *Australorbis glabratus*. *Exper. Parasitol.*, **2:** 242–257.
15. Weinbach, E. C., 1957. Biochemical basis for the toxicity of pentachlorophenol. *Proc. Nat. Acad. Sci. U. S.*, **43:** 393–397.
16. Weinbach, E. C., and Nolan, M. O., 1956. The effect of pentachlorophenol on the metabolism of the snail *Australorbis glabratus*. *Exper. Parasitol.*, **3:** 276–284.
17. Sharaf El Din, H. and El Nagar, H., 1955. Control of snails by copper sulphate in the canals of the Gezira irrigated area of the Sudan. *J. Trop. Med. & Hyg.*, **58:** 260–263.
18. El Nagar, H., 1958. Control of schistosomiasis in the Gezira, Sudan. *J. Trop. Med. & Hyg.*, **61:** 231–235.
19. Alcay, L., Marill, F., and Musso, J., 1942. Le foyer de bilharziose urinaire de Saint-Aime-de-la-Djidiouia (Department d'Oran, Algérie). *Arch. Inst. Pasteur Algérie*, **20:** 39–99.
20. Watson, J. M., 1951. Studies on bilharziasis in Iraq. Part VI. Seasonal variation of the vector snail, *Bulinus truncatus*. *J. Fac. Med. Baghdad*, **15:** 33–69.
21. Cridland, C. C., 1957. Ecological factors affecting the numbers of snails in permanent bodies of water. *J. Trop. Med. & Hyg.*, **60:** 250–256.
22. Gaud, J., 1958. Rythmes biologiques des mollusques vecteurs des bilharzioses. *Bull. World Health Organ.*, **18:** 751–769.

TRANSACTIONS OF THE AMERICAN SOCIETY OF TROPICAL MEDICINE AND HYGIENE

Eighth Annual Meeting

Indianapolis, Ind., October 29–31, 1959

OFFICERS

President—Lewis W. Hackett
President-Elect—Willard H. Wright
Vice-President—Oliver R. McCoy
Secretary-Treasurer—Rolla B. Hill
Editor—Martin Frobisher
Editor pro tem.—Martin D. Young

COUNCILORS

W. C. Reeves
J. M. Henderson
P. F. Russell
M. Hertig
L. A. Stauber
J. A. Scott
J. E. Smadel
J. E. Larsh, Jr.

FORMER PRESIDENTS OF THE SOCIETY

Martin D. Young 1952
William A. Sodeman 1952–53
Frederick J. Brady 1953–54
William A. Taliaferro 1954–55
Asa C. Chandler 1955–56
Justin M. Andrews 1956–57
Donald L. Augustine 1957–58

MINUTES OF THE ANNUAL COUNCIL MEETING

The annual business meeting of the Council was held October 28, with President Hackett presiding. All the Council members were present with the exception of P. F. Russell, and in addition Drs. Brady and Salisbury were in attendance.

The minutes of the seventh annual meeting, held in Miami Beach, were approved as published in the *Journal* for March 1959.

The Secretary's report was presented as follows:

This report covers the period of the fiscal year, from October 1, 1958 through September 30, 1959. Highlights of the year were (1) publication of a new Membership List; (2) work on a revised edition of Health Hints for the Tropics; and the resignation of the Editor, Martin Frobisher with Martin D. Young being appointed Editor pro tem.

Annual meetings. The seventh annual meeting was held at Miami Beach, November 5–8, 1958, with over 300 members in attendance. Dr. Ralph Jones Jr. and Dr. J. Walter Beck were the Chairman and Co-Chairman respectively, of the Local Committee in charge of arrangements.

The printed programs for the 1959 meeting were mailed to all members on August 25.

The Council has voted to hold the 1960 meeting jointly with the American Society of Parasitologists in Los Angeles, Nov. 2–5. The Council has also approved meeting in Washington in 1961, and in Chicago, again with the American Society of Parasitologists, in 1963. The meeting place for the 1962 meeting has not yet been decided upon.

Membership. At the end of the year the Society had enrolled, 1,086 Active Members in good standing, plus 2 Life Members, 13 Emeritus Members and 12 Honorary Members, making a total of 1,113, which is two more than in the preceding year. In addition, there were 73 members in arrears of dues for 1959, 31 in arrears for 1958 and 26 whose dues were unpaid for 1957, 1958 and 1959. These last will be dropped from our rolls at the end of the calendar year. Adding these

130 members in arrears of dues for one or more years, to the 1,113 in good standing, gives a total membership on our books of 1,243.

Included in the above total are 74 new members elected in 1959, whom we welcome into the Society. There were 70 new members in the previous year.

With much regret, we record the death of the following valued members:

ARTHUR C. BAKER
JEFF W. CHAPMAN
ANTONIO L. EJERCITO
WILLIAM H. GORDON
R. B. H. GRADWOHL
HERBERT G. JOHNSTONE
RALPH W. NAUSS
ANGELO M. PERRI
CORNELIUS P. RHOADS
GEOFFREY W. RAKE
T. K. ROUDEBUSH
GEORGE W. STRODE

Thirty-two members submitted their resignation during the year, and 26 members, in arrears of dues for three years, were dropped in accordance with Art. XI of our By-Laws.

Letters to the Council. Five letters were sent out during the year, dealing with various aspects of the affairs of our Society. These included notification of the resignation of our Editor following major surgery; a ballot on Clyde Swartzwelder, who was elected to the Editorship, but for personal reasons was obliged to resign shortly thereafter; ballot and approval of Washington as the site of the 1961 meeting; and the appointment of a new Chairman and another member of the Bailey K. Ashford Award Committee, following the resignation of Martin D. Young. William W. Frye was made the Chairman and Henry E. Meleney the new member. An agenda for the annual Council meeting was also sent to the Council members.

Other activities. As directed by the Council at the last annual meeting, a new Membership List was prepared, printed and sent to all members with the April issue of Tropical Medicine and Hygiene News. Ours is a very mobile group, and already there have been some 200 changes of address since the appearance of the 1959 list.

Revised Edition of Health Hints for the Tropics. The previous edition being nearly exhausted, a new edition of this popular pamphlet is being prepared under the direction of Harold W. Brown.

Activities in connection with the various Committees are reported under the appropriate headings.

Respectfully submitted,
ROLLA B. HILL,
Secretary

The report of the Secretary was accepted as read.

The Treasurer's report was presented as follows:

As is shown in the auditor's report, given below, the treasury is in a much better financial condition than it has been for several years. We ended the financial year with $4,566.93 cash in hand, as compared with $1,913.22 for the previous period. This favorable situation is due largely to the increase in dues which took effect this year. Our total receipts for the year were $15,044.07, and expenses $12,390.36.

Our total assets were $29,148.47, an increase of approximately $2,400. Most of this is in Government Savings Bonds, the interest on which is available for current expenses and for the publication of our Transactions, and for Review articles. The amount available for these purposes at the end of the year was $1,226.08.

The Journal Fund, which can be used for extra pages for the Journal, remained at $1,904.38.

Excess of receipts over expenses in connection with the printing of the Journal, as reported by the publisher, amounted to $1,576.76 for the calendar year ending December 31, 1958. The estimated excess for the current year is a good deal less. The amount received from the Journal was sufficient to pay for the Editorial expenses, which totaled $1,141.97.

On the other hand, expenditures for the publication of Tropical Medicine and Hygiene News were $3,027.54, which exceeded receipts by about $500, and as a consequence the cash balance at the end of the year was reduced by a like amount. This deficit was due principally to the cost of printing the 1959 Membership List.

Respectfully submitted,
ROLLA B. HILL,
Treasurer

October 13, 1959

The Officers and Councilors
The American Society of
Tropical Medicine and Hygiene
Miami, Florida

Gentlemen:

I have examined the financial records of *The American Society of Tropical Medicine and Hygiene*, Miami, Florida, and *Tropical Medicine and Hygiene News*, Bethesda, Maryland, for the fiscal year ended September 30, 1959 and now submit as my report the exhibits, schedules and comments which follow.

SCOPE OF EXAMINATION

My examination was made in accordance with generally accepted auditing standards, and accordingly, included such tests of the accounting records and other auditing procedures as I considered necessary in the circumstances to enable me to render an independent accountant's opinion on the fairness of the representations in the statements showing the assets and liabilities of the organization at September 30, 1959, and its operating results for the fiscal year then ended. Auditing procedures employed included those set forth in the following paragraphs:

The bank balances were confirmed by direct communications with the depositories. All cash receipts and disbursements were traced through the bank accounts. All other current assets, with the exceptions of (1) inventory of "Health Hints for the Tropics" included at a cost of $173.60 and (2) the account receivable of $1,478.35 due from Williams and Wilkins as *estimated* profit on 1959 issues of the JOURNAL, were either computed by me or otherwise satisfactorily verified.

The United States Savings Bonds in the amount of $20,385.50, stated at cost, were inspected at the Coconut Grove Bank and found to be in the name of the Society.

There were no changes in fixed assets during the fiscal year.

I made sufficient tests of the operating accounts to satisfy myself that accepted accounting practices had been followed and that the transactions for the fiscal year had been properly and consistently classified. As to the receipt of membership dues, it was not practicable under the scope of the engagement to extend the review beyond ascertaining that the receipts, as reported on the books, had been deposited in the Society's bank account.

ACCOUNTANT'S OPINION

In my opinion, the accompanying balance sheets, and related cash receipts statements, fairly present the assets and liabilities of THE AMERICAN SOCIETY OF TROPICAL MEDICINE AND HYGIENE, Miami, Florida, and TROPICAL MEDICINE AND HYGIENE NEWS, Bethesda, Maryland, at September 30, 1959, resulting from cash transactions, and the income collected and the expenses disbursed for the fiscal year then ended, and are presented on a basis consistent with that of the preceding year.

W. KEITH WHEELING
Certified Public Accountant

Exhibit A

The American Society of Tropical Medicine and Hygiene
Miami, Florida

COMPARATIVE BALANCE SHEETS

September 30, 1959 and 1958

Assets

	September 30, 1959		*September 30, 1958*	
Current Assets:				
Cash in bank (*Exhibit B*)	$4,566.93		$1,913.22	
Accounts receivable (Note 1)	1,478.35		1,311.68	
Interest receivable, U.S. Savings Bonds (*Exhibit A*, Schedule 1)	179.40	$6,224.68	179.40	$3,404.30
Investment Assets:				
U.S. Savings Bonds, at cost (*Exhibit A*, Schedule 1) (Note 2)				
Series J (Maturity Value $275.00)	$198.00		$198.00	
Series K (2.76%)	20,000.00		20,000.00	
Series E	187.50			
	$20,385.50		$20,198.00	
Tropical Medicine and Hygiene News (*Exhibit C*)	2,066.05	22,451.55	2,629.50	22,827.50
Fixed Assets:				
Office equipment		472.24		472.24
Total Assets		$29,148.47		$26,704.04

Liabilities and Net Worth

Current Liabilities:				
Payroll taxes and withheld wages due		$138.00		$45.00
Deferred Credits to Income:				
Prepaid dues		196.25		96.00
Total Liabilities		$334.25		$141.00
Net Worth:				
Retained income				
Balance, September 30, 1958 and 1957	$26,090.80		$24,600.61	
Increase for fiscal years ended September 30, 1959 and 1958	2,251.18		1,490.19	
Balances, September 30, 1959 and 1958 (Note 3)	$28,341.98		$26,090.80	
Investment in office equipment	472.24	28,814.22	472.24	26,563.04
Total Liabilities and Net Worth		$29,148.47		$26,704.04

Note 1. The account receivable represents the estimated profit from the 1959 publication of four issues of *The American Journal of Tropical Medicine and Hygiene* by the Williams and Wilkins Company.

Note 2. Interest on U. S. Savings Bonds, Series J, is available for current expenditures; interest on Series K and E bonds is available for reviews in the *Journal* and for publishing abstracts of papers on the program.

Note 3. Of the $28,341.98 in retained income as of September 30, 1959, $4,826.02 is unappropriated as of that date, the remainder having been appropriated for:

Extra pages for the *Journal*		$1,904.38
Life membership fees of:		
(A) Dr. Charles A. Werner invested in U.S. Savings Bonds, Series J	$198.00	
(B) Dr. Etta Mae MacDonald invested in U.S. Savings Bonds, Series E	187.50	385.50
Investment in U.S. Savings Bonds, Series K, the interest on which is available for reviews in the *Journal* and for publishing abstracts of papers on the program		20,000.00
Interest on Series K Bonds collected to September 30, 1959, less expenditures to date (See Exhibit A, Schedule 1)		1,226.08
Total Appropriated Retained Income		$23,515.96

Exhibit A, Schedule 1

The American Society of Tropical Medicine and Hygiene
Miami, Florida

INVESTMENTS IN U.S. SAVINGS BONDS

September 30, 1959

Serial Number	*Date of Purchase*	*Cost*	*Maturity Value*	*Accrued Interest*
Series J				
Q 21413 J	6-29-54	$18.00	$25.00	
Q 21414 J	6-29-54	18.00	25.00	
Q 21415 J	6-29-54	18.00	25.00	
C 93631 J	6-29-54	72.00	100.00	
C 93632 J	6-29-54	72.00	100.00	
Total investment Series J		$198.00	$275.00	

Series K				
M 91038 K	4-14-53	$1,000.00	$1,000.00	$13.80
M 91039 K	4-14-53	1,000.00	1,000.00	13.80
M 91040 K	4-14-53	1,000.00	1,000.00	13.80
M 91041 K	4-14-53	1,000.00	1,000.00	13.80
M 195142 K	6-29-54	1,000.00	1,000.00	9.20
V 26490 K	6-29-54	5,000.00	5,000.00	46.00
V 26514 K	7-9-54	5,000.00	5,000.00	34.50
V 26515 K	7-9-54	5,000.00	5,000.00	34.50
Total investment Series K		$20,000.00	$20,000.00	$179.40
Series E				
L 461445756 E	12-9-58	$37.50	$50.00	
C 357038215 E	12-9-58	75.00	100.00	
C 357038216 E	12-9-58	75.00	100.00	
Total investment Series E		$187.50	$250.00	
Total investments in U.S. Savings Bonds		$20,385.50	$20,525.00	

Notes:

(1) The Series E bonds were purchased during the fiscal year ended September 30, 1959 with funds provided by a life membership purchased by Dr. Etta Mae MacDonald.

(2) All bonds are in the name of The American Society of Tropical Medicine and Hygiene and are kept in the society's safety deposit box in the Coconut Grove Bank, Miami, Florida, where they were inspected on September 30, 1959 in company with the Secretary-Treasurer, Dr. Hill.

(3) The interest earned on the Series E and Series J bonds is available for current expenditures. However, no interest will be received until maturity dates unless the bonds are redeemed prior to maturity. The bonds increase in redemption value each year. As of September 30, 1959, the redemption value of the Series E bonds was $189.00 and the Series J bonds was $220.00.

(4) Interest on Series K bonds is available for reviews in the *Journal* and for publishing abstracts of papers on the program. Interest available for this purpose is summarized below:

Total interest available as of September 30, 1958	$674.08
Interest collected during fiscal year	552.00
Total interest available	$1,226.08
Expenditures during current period	—
Balance of interest available as of September 30, 1959	$1,226.08

Exhibit B

The American Society of Tropical Medicine and Hygiene

Miami, Florida

COMPARATIVE STATEMENTS OF CASH RECEIPTS AND DISBURSEMENTS

For the Fiscal Year Ended September 30, 1959 and 1958

	Year Ended September 30, 1959		*Year Ended September 30, 1958*	
Beginning cash balances, September 30, 1958 and 1957		$1,913.22		$3,359.64
Receipts:				
Membership dues:				
1960	$196.25		$96.00	
1959	10,701.00		8,625.50	
1958	182.50		210.50	
Prior years	22.00	11,101.75	28.00	8,960.00

The American Journal of Tropical Medicine and Hygiene profit for calendar years 1958 and 1957		1,576.76		27.57
Profits from annual meetings at Miami Beach, Florida (1959) and Philadelphia, Pennsylvania (1958)				
Share of income from exhibits	$501.01		$339.97	
Registrations	576.00		229.17	
Refunds on advances to local committees on arrangements	54.07			
Fee paid to society by Sunshine Tours	40.00	1,171.08		569.14
Interest earned on U.S. Savings Bonds (*Exhibit A*, Schedule 1)		552.00		552.00
Miscellaneous transactions in connection with dues and subscriptions		17.00		40.00
Donation for next annual meeting received from Winthrop Products, Inc.		500.00		
Publication of *Tropical Medicine and Hygiene News*				500.00
Payment received on a life membership from E. M. MacDonald		100.00		100.00
Use of stencils by Lea and Febiger				24.00
Refund on express charges from *News* envelopes		25.48		
Total cash available		$16,957.29		$14,132.35
Disbursements:				
Office expenses: (*Exhibit B, Schedule 1*)				
Editorial (Note 1)	$1,141.97		$3,283.46*	
Secretary-Treasurer	3,651.16		1,912.44	
Total office expenses	$4,793.13		$5,195.90	
Williams and Wilkins for subscriptions to the *Journal*	6,636.00		6,678.00	
Publication of abstracts (*Exhibit A, Schedule 1*)	—		345.23	
Purchase of Series E Bonds with life membership purchased by Dr. MacDonald	187.50			
Advance to local committee for next annual meeting	500.00			
Expenses of annual meeting held at Miami Beach, current year	273.73	12,390.36		12,219.13
Ending cash balances September 30, 1959 and September 30, 1958		$4,566.93		$1,913.22
Consisting of: Cash in bank		$4,566.93		$1,913.22

Note 1. *The editorial expenses for the fiscal year ended September 30, 1957 were *not* reimbursed to Dr. Hackett until October, 1958. Of the figure $3,283.46, $2,010.70 represents editorial expenses of the fiscal year ending September 30, 1957 and $1,272.76 represents editorial expenses of the fiscal year ending September 30, 1958. (For an itemized comparison of the editorial expenses for the two fiscal periods, see Exhibit B, Schedule 1.)

Exhibit B, Schedule 1

The American Society of Tropical Medicine and Hygiene
Miami, Florida

OFFICE EXPENSES

For the Fiscal Years Ended September 30, 1959 and 1958

Expenses	*Year Ended September 30, 1959*		*Year Ended September 30, 1958*	
	Editorial	*Secretary-Treasurer*	*Editorial*	*Secretary-Treasurer*
Secretarial assistance	$668.49	$2,487.00	$769.54	$935.00
Honorarium, assistant to editor			75.00	
Travel and meetings	191.04	22.30	81.39	11.58
Postage	188.78	247.53	229.92	187.25
Printing	5.04	450.80		352.30
Office Supplies	47.25	52.96	101.33	74.14
Telephone and telegraph	15.29	19.38	15.58	8.37
Auditing fee		175.00		175.00
Medals		50.38		22.38
Freight and express		23.41		32.95
Corporate registration		30.00		30.00
Contribution to National Society for Medical Research		25.00		25.00
Miscellaneous	26.08	67.40		58.47
Total office expenses	$1,141.97	$3,651.16	$1,272.76*	$1,912.44

* Actual cash disbursed for editorial expenses for the fiscal year ended September 30, 1958 totalled $3,283.46. However, $2,010.70 of this total was disbursed in October of 1958 and actually covered fiscal year 1957 expenses. Therefore, as shown in last year's report, the actual 1958 fiscal year editorial expenses only of $1,272.76 are shown above to give a meaningful comparison between the two fiscal periods.

Exhibit C

Tropical Medicine and Hygiene News
Bethesda, Maryland

COMPARATIVE BALANCE SHEETS

September 30, 1959 and 1958

Assets

	September 30, 1959	*September 30, 1958*
Current Assets:		
Cash on hand and in bank (*Exhibit D*)	$1,746.13	$2,193.91
Accounts receivable	125.00	132.00
Stamps and postage deposit on metered mail	21.32	15.97
Inventory ("Health Hints for the Tropics")	173.60	287.62
Total Assets	$2,066.05	$2,629.50

Net Worth

Retained Earnings:		
Balances September 30, 1958 and 1957	$2,629.50	$1,828.73
Increase for years ended September 30, 1959 and 1958	(563.45)	800.77
Total Net Worth (*Exhibit A*)	$2,066.05	$2,629.50

Exhibit D

Tropical Medicine and Hygiene News
Bethesda, Maryland

COMPARATIVE STATEMENTS OF CASH RECEIPTS AND DISBURSEMENTS

For the Fiscal Years Ended September 30, 1959 and 1958

	September 30, 1959		*September 30, 1958*	
Beginning cash balances, September 30, 1958 and 1957		$2,193.91		$1,598.41
Receipts:				
Advertising	$1,250.00		$1,375.00	
Sale of supplements	711.76		1,846.92	
American Foundation for Tropical Medicine	618.00	2,579.76	721.00	3,942.92
		$4,773.67		$5,541.33
Disbursements:				
Printing of *Tropical Medicine and Hygiene News* and membership list (Note 1)	$1,294.63		$888.00	
Printing of "Health Hints for the Tropics"			851.34	
Copy preparation of *News* and list (Note 1)	658.04		547.00	
Postage and express	228.70		164.78	
Office supplies	197.28		66.37	
Secretarial help	343.00		141.47	
Deposits on postage meter account (Note 1)	185.00		100.00	
Travel expenses (editor)	117.70		86.53	
Transferred to American Society of Tropical Medicine and Hygiene	—		500.00	
Miscellaneous	3.19	3,027.54	1.93	3,347.42
Ending cash balances, September 30, 1959 and 1958		$1,746.13		$2,193.91
Consisting of:				
Cash in Bank of Bethesda		$1,705.09		$1,632.24
Undeposited checks (Deposited October 2, 1958)		—		559.27
Cash on hand		41.04		2.40
Total cash on hand and in bank (*Exhibit C*)		$1,746.13		$2,193.91

Note 1. The increased disbursements indicated in 1959 in these accounts were made in connection with the preparation of a new membership list during the fiscal period ended September 30, 1959.

The Treasurer's report was accepted as submitted.

The Report of the Editor of the *American Journal of Tropical Medicine and Hygiene* was presented as follows:

Although the Editorial staff of the Journal and, as a consequence, The President and Secretary of the Society, had a difficult year due to the illness of the Editor-in-Chief, the Journal itself continued very much as formerly. Volume 8, 1959, contained 99 titles (not including 34 Book Reviews) in 731 pages, and also a supplement of 66 pages in the March issue. This compares with 99 titles and 32 Book Reviews in 668 pages in Volume 7, 1958. Volume 8 is the first volume completely in the double-column format, which reduces the number of pages by about one-third. Some slight changes in style have been undertaken, especially in form of reference citations.

Due to the disability of the Editor, beginning in February, he felt it his duty to the Journal to resign as Editor in April. That the Journal continued to operate without interruption, was due at first solely to the devoted efforts of the Editor's secretary, Mrs. Alice P. Snyder, and Mrs. Frobisher. Afterward it continued because Dr. Martin D. Young became Editor-in-Chief pro tem., with the Editor later assisting as Issue Editor and Book Review Editor. Together these carried on, bringing out the September and November issues of Volume 8 and the January

issue of Volume 9 under joint auspices. The Journal is now as flourishing as ever.

The financial statement of the Journal for 1959, prepared by Williams and Wilkins, and estimated as is usual at this time of year, is within the range of normal variation and compares favorably with that of last year. The most important items, namely cash on hand at the end of the year, are $1,576.76 for 1958 (Volume 7) (actual) and $1,400 for 1959 (Volume 8) (estimated)).

A tabulation of papers received since the last annual report, their subject categories, and their disposition, is shown in Table 1. This tabulation clearly indicates an increase of papers in the field of virus diseases. It is of interest to note, with a view to future prospects of the Journal as a medium for continued publication in the field of tropical medicine, that many of the papers have been supported by research grant funds. This suggests that these grants have stimulated research in the field of tropical medicine. On the other hand, the number of journals to publish these researches has not increased.

During the year the Editor's office needed a bound set of the Journal for reference, etc. It appeared that the Society did not possess a bound set of its own Journal. Upon requesting the publishers to furnish a set to be kept in the Editor's office, it was found that all back volumes of the Journal prior to 1954, as well as the 1958 volume and all of the old American Journal of Tropical Medicine, had been sold to the Johnson Reprint Company. Dr. Hackett, therefore, kindly gave his back volumes of the Journal to the Society so that the Editor's office could have a bound set.

TABLE 1

Categories and disposition of manuscripts received during the period November 1, 1958, to October 21, 1959 (i.e., *between annual reports*)

Category*	Accepted	Rejected	In Process	Total	Subtotal
P —Protozoology	18	11	6	35	
H —Helminthology	21	5	5	31	
HP —Hel. & Prot.	4	2	0	6	72
V —Virology	29	2	2	33	33
E —Entomology	9	2	1	12	12
B —Bacteriology	5	3	2	10	
M —Malacology	3	1	0	4	
My —Mycology	1	0	0	1	
N —Nutrition	1	0	0	1	
Ph —Pharmacology	0	1	0	1	17
Misc—Miscellaneous	6	4	0	10	10
	97	31	16	144	144

* In the treatment of disease, manuscripts are shown under etiological agent; in control, under vector.

In closing, the Editors wish to express their heartfelt thanks to the Editorial Board, as well as to Dr. Hackett and Dr. Hill, without whose help the Journal could not have continued to operate regularly and to maintain the high and honored position which this publication has always held among scientific publications the world over.

Respectfully submitted,
MARTIN FROBISHER, *Editor*
MARTIN D. YOUNG,
Editor-in-Chief, pro tem.

By motion, duly seconded and passed, the Council expressed its gratitude to the people who aided Dr. Frobisher, and to Dr. Frobisher himself and Dr. Young for their efficient work under difficult circumstances.

The Report of the Editor of Tropical Medicine and Hygiene News was presented as follows:

Six issues of *Tropical Medicine and Hygiene News* were published on schedule. The *News* went to all members of the Society and to between 600 and 700 interested non-members, most of whom live outside the United States. The editorial policy remains essentially unchanged and only minor changes in format were made.

There was a decline in net worth of $563.45. This undesirable event is attributable both to a decline in revenue and an increase in operating costs. Continuing a long-term trend, there was a decrease in revenue from advertising, which is our chief source of income. This trend is a cause for grave concern. Unless this trend can be reversed the *News* will continue in a precarious financial condition. Special efforts to increase advertising revenue are urgently needed.

Sale of "Health Hints for the Tropics" continued at a satisfactory level. Income from this venture made up in part for the large loss in printing and distributing the *News*.

The American Foundation for Tropical Medicine continued a long-standing and mutually profitable arrangement under which the *News* is printed especially for their mailing lists.

In April the Membership List was reissued and sent to all members of the Society. The List was made up by the Secretary-Treasurer and set up

and printed in Washington. The cost of the List was charged to the *News* account, as usual. The total cost of the Membership List cannot be stated exactly, since part of the cost is buried in the running expenses of both Society offices. However, the cost is conservatively estimated to be $400.00.

The Editor of the *News* wishes to thank the Secretary-Treasurer for his consistent friendly help and advice.

Suggestions as to how the *News* can be improved will be welcome.

LOUIS J. OLIVIER,
Editor

The report of the Editor of the *News* was accepted, with a vote of thanks and appreciation for his efficient work.

REPORTS OF COMMITTEES

Bailey K. Ashford Award. Dr. Martin D. Young, Chairman, resigned from the Committee when he learned that one of his colleagues had been nominated for the Award. Prior to his resignation, Dr. Leighty of The Eli Company, sponsors of the Award, agreed to the suggestion of the Committee that the age limit be increased to 40 years.

Dr. W. W. Frye was appointed Chairman of the Committee, and Dr. Henry Meleney became the third member, the second member being Dr. Clay G. Huff.

After due consideration of several nominees, the Committee selected Dr. Geoffrey M. Jeffery to receive the Award.

Charles Franklin Craig Lecture. It was the unanimous opinion of our Committee that Fred L. Soper be invited to present the Charles Franklin Craig Lecture. Dr. Soper kindly accepted.

Honorary Membership. Your Committee has continued the policy of the Society and has not made any recommendations for Honorary Membership for this year.

Membership. Your Committee has examined the applications and credentials of 74 prospective members of the Society, and has approved all of them.

The total membership on September 30, 1959, was 1,243, compared with 1,131 a year ago. Continuing the present Society policy, the Committee has made no concerted effort to obtain new members.

Nominating. Your Committee drew up a slate of candidates for the various offices for 1959–60, which was submitted to the Secretary for preparation of the official ballot. Your Committee respectfully suggests that in the future prospective nominees be advised and their acceptance secured before they are actually placed on the ballot.

Program. In response to the call for papers, an unusually large number of requests for a place on the program of the Indianapolis meeting were received. Because of the limit on the number which could be accomodated, it was necessary to decline several applications. The Committee spent a good deal of time in making the selection of papers accepted.

Public Relations. On February 6, Dr. Brady informed the Secretary of the Society that the Sub-committee on Reorganization and International Organizations of the Committee on Government Operations, of which Senator Humphrey is Chairman, was soliciting the views of scientific organizations having an interest in international health, and suggested that this Society offer to cooperate with the Sub-committee, especially since much of the material to be developed will deal with tropical medicine.

Actually, the Secretary had received a request for our views from Senator Humphrey in December, together with a number of the documents already published by the Committee and the Sub-committee. This material was received from the Secretary of the Society at the end of December and your Committee thereupon prepared a statement setting forth the present and past activities of the Society, and suggesting several areas of potential interest to the Sub-committee which might help to develop the field of international health. These included:

1. Medical intelligence and information.
2. Exchange of student and research personnel.
3. Training of foreign students in American medical schools and public health institutes.
4. Graduate training in public health and tropical medicine.
5. International medical meetings.
6. Program for eradication of malaria.
7. Continued support of the World Health Organization.

This statement was sent on January 20, and acknowledged with thanks on January 29.

Late in September the Society was informed by Senator Humphrey of future publications of the Sub-committee—they have already published 4 pamphlets—while at the same time he invited our suggestions on the role of American Medical organizations as such, in international medical groups. This invitation has not yet been replied to.

All of the above reports were accepted as presented, with thanks and appreciation to the members of the various committees for their devoted interest in the affairs of the Society.

It was also voted that the *ad hoc* Public Relations Committee be continued.

REPORT OF REPRESENTATIVES TO ORGANIZATIONS

National Research Council, Division of Medical Sciences. Submitted by Leon Jacobs.

I am enclosing the minutes of the Annual meeting of the National Research Council, 20 March, 1959. The items on p. 12 et seq. may be of special interest to the Council of the Society. Since the Hill bill, proposing a new National Institute of International Health, has not yet been passed, the discussion concerning how the Institute would function is somewhat nebulous. Policy questions concerning the control of international operations are still unresolved. Our Society, however, can anticipate that if any such Institute is established, it may represent an opportunity for the Society to assume a consultant role or to exert its influence in relation to international health problems. Similarly, any tropical operations in the International Health and Medical Research Year, proposed by Senator Humphrey's resolution, may be expected to involve our Society.

I am also enclosing a copy of the Annual Report of the Division of Medical Sciences, NRC. I should like to point out, on p. 16, what I consider a very significant note on infectious diseases in general. It is most important that studies on the fundamental biology, pathogenesis, and immunologic phenomena relating to micro-organisms be continued despite the availability of drugs against them and despite their lessened statistical importance in some geographic areas. I should like to recommend to the Council of the Society that it consider communicating its interest in this field to the Chairman of the Division of Medical Sciences, and supporting the recommendation of the Committee on Infectious Diseases and Chemotherapy, NRC, that a new committee devoted to these basic problems be established, and nominating some Society members.

Your attention is also directed to pp. 12–14 concerning the survey of the field of tropical medicine. Since Dr. Wright is our President-Elect, he will undoubtedly communicate to the Council on these activities.

The report was accepted, with thanks to Dr. Jacobs for so ably representing the Society in the Division of Medical Sciences.

Dr. Willard H. Wright then gave a brief report of his activities as Director of the Commission on Medical and Hygienic Problems of the Tropics of the National Research Council. Since he assumed his post on January 1, 1959, he has been assembling his staff and preparing the bases of the studies which the Commission has now undertaken.

American Foundation for Tropical Medicine. Report submitted by Dr. E. I. Salisbury. The Liberian Institute of the Foundation continues to grow in importance. In 1959 the resident staff was composed of 12 persons. It is anticipated that in the future the number of visiting scientists requesting facilities to work at the Institute will increase. A request has already been received from the Tropensinstitut, Hamburg, for permission to send a pathologist to the Institute for a one-year period. W.H.O. also wishes to have one of their entomologists at the Institute for a year.

Two types of financial support are available to the Foundation and Institute, unrestricted support, and restricted support, for specific projects. To house the increasing number of foreign staff members, three new houses are nearing completion at Harbel. A number of staff members are being sought to assist in the work now under way or in contemplation at the Institute.

Studies under way at the Institute include in part: studies on the bionomics of snail hosts of human schistosomiasis; malaria in the chimpanzee; trypanosomiasis control. It is hoped to establish a breeding chimpanzee colony in the near future. A Field Station at Voinjama in Northern Liberia has been established, which is now used for the trypanosomiasis studies.

OTHER OLD BUSINESS

Election of Officers and Councilors for 1959-60. The slate submitted by the Nominating Committee was put into ballot form and mailed to all voting members in March. The 261 votes received were tabulated by the Tellers appointed by the President, with the following result:

President-elect.....WILLIAM W. FRYE
Vice President.....G. ROBERT COATNEY
Councilors.....ARNOLDO GABALDON
HARRY MOST

NEW BUSINESS

Election of an Editor of Society Publications. The Council cast a unanimous ballot for Paul C. Beaver as Editor, and authorised him to appoint one or more Associate Editors.

Election of a Secretary-Treasurer. The Council re-elected Rolla B. Hill as Secretary-Treasurer.

Contribution to the National Society for Medical Research. It was voted to again contribute $25.00 toward the support of this Society.

Committee Appointments. The Council elected the following members to serve on the Nominating

Committee for 1959–60: T. H. Weller, J. M. Henderson, and Leon Schmidt.

The Council appointed Frederick J. Brady to serve on the Walter Reed Medal Committee for a term of 6 years.

It was announced that the Joseph Augustin LePrince Award is due to be given in 1960.

Drs. W. W. Cort and Robert A. Lambert were elected Emeritus members.

The President was authorized to appoint such *ad hoc* Committees as he deems necessary and advisable.

Support of the Annual Meetings by Commercial Houses. During the year it was suggested by one of the pharmaceutical houses that the Society might seek wider support from commercial firms for the annual meetings, and after discussion this was agreed to in principle, as being in the best interests of the supporters and the Society itself.

It was agreed that a Symposium be held at the 1960 meeting, and W. C. Reeves was appointed to organize it.

Problems in Tropical Medicine. Dr. Stauber presented selections from a booklet "100 Problems in Environmental Health," and at the instance of the Tropical Medicine and Parasitology Section, Division of Research Grants, National Institutes of Health, inquired whether it might be advisable for the Society to publish a similar booklet on Tropical Medicine. No action was taken at this time.

There being no further business, the meeting adjourned.

BUSINESS MEETING

The business meeting was held on Saturday, October 31, with President Hackett in the chair.

The annual reports of the Secretary and Treasurer were presented, as well as the report of actions by the Council. The results of the ballot for officers and councilors was announced, and Willard H. Wright, the new President took the chair.

The report of the Resolutions Committee was read by Dr. Hunter, and adopted by the Society.

Dr. Hunter moved that a series of suggestions be formulated for presentation of papers and accompanying slides, to be sent out when papers are called for. The motion was carried.

The suggestion was made from the floor that abstracts of papers to be presented be prepared, and published either before or after the meeting. The Council has voted not to publish abstracts, since there are numerous disadvantages thereto. No change is contemplated at the present time.

Report of the Resolutions Committee (John L. Larsh, Jr. and George W. Hunter, III)

1. It is requested that the Secretary of the Society be instructed to write and send resolutions of sympathy to the families of deceased members of the American Society of Tropical Medicine and Hygiene. During the current year the Society has lost Arthur C. Baker, J. W. Chapman, A. L. Ejercito, E. J. de Beer, W. N. Gordon, R. B. H. Gradwohl, H. G. Johnstone, R. W. Nauss, Angelo M. Perri, G. W. Rake, C. P. Rhoads and T. K. Roudebush.

2. To Fred L. Soper we extend the Society's sincere thanks and highest esteem for his presentation of the Twenty-Fourth Annual Charles Franklin Craig Lecture, "Thoughts on Malaria Eradication, 1959."

3. The Society expresses its appreciation to the organizations and individuals who have served as hosts, or otherwise furnished helpful assistance, for the Eighth Annual Meeting of the Society held in Indianapolis, Indiana, October 28–31, 1959.

A. The officers and members of the Society are deeply appreciative of the assistance and hospitality extended by the Indiana University School of Medicine and by members of its faculty and staff. THEREFORE BE IT RESOLVED that the thanks of the Society be expressed to the Dean, Dr. John D. Van Nuys and, through him, to the Faculty and Staff.

B. The Society appreciates the role played by the sponsors in assuring the success of the meeting. THEREFORE BE IT RESOLVED that the thanks of the Society be extended to: Burroughs Wellcome and Company (U.S.A.) Inc., Eli Lilly and Company, Pitman-Moore Company, Upjohn Company, Winthrop Products, Inc., and the Indiana State Board of Health.

C. The assistance and cooperation of the staff of the Claypool Hotel contributed to the success of the meeting. THEREFORE BE IT RESOLVED that the thanks and appreciation of the Society be expressed to Mr. W. Bryan Karr, Manager, and, through him, to members of the hotel staff.

D. The Indianapolis Convention and Visitors Bureau supplied secretarial help and other assistance of great value. THEREFORE BE IT RESOLVED that the thanks and appreciation of the Society be expressed to Mr. Joseph J. Cripe, Manager of the Bureau.

E. The planning of the Local Committee on

Arrangements was an essential contribution to the success of the meeting. THEREFORE BE IT RESOLVED that the thanks and appreciation of the Society be expressed to Dr. Max C. McCowen and Dr. W. A. Summers, Co-Chairmen, and Committee members as follows: Drs. M. C. Brandt, M. E. Callender, F. A. Ehrenford, J. G. Evans, H. L. Feldman, R. B. Galloway, J. B. Hammond, W. H. Headlee, K. L. Harper, S. H. Hopper, R. W. Newman, C. L. Rose, and R. F. Shumard.

F. The hospitality sessions were a most important contribution to the pleasure of the individual members and guests and the success of the meeting as a whole. THEREFORE BE IT RESOLVED that the thanks and appreciation of the Society be extended to Eli Lilly and Company and The Pitman-Moore Company for providing these sessions.

G. The commercial exhibits added materially to the interest of the meeting. THEREFORE BE IT RESOLVED that the thanks and appreciation of the Society be expressed to Mrs. Herma H. Hoefler, who served as Chairman of the Commercial Exhibits Arrangements, and through her to the commercial exhibitors. She also had charge of the Scientific Exhibits.

H. The Scientific Exhibits were of outstanding interest. THEREFORE BE IT RESOLVED that the thanks and appreciation of the Society be expressed to the individuals who contributed these exhibits, through their institutions as follows: the International Cooperation Administration, Washington, D. C.; Cornell University Medical College, New York, N. Y., and Calcutta School of Tropical Medicine, Calcutta, India; National Institutes of Health, Memphis, Tenn.

I. The interest and planning of the Committee for Ladies' Entertainment contributed greatly to the pleasure of the ladies attending the meeting. THERFORE BE IT RESOLVED that the Society express its thanks and appreciation to Mrs. M. C. McCowen and Mrs. C. L. Rose, Co-Chairmen, and through them to the other members of the Committee: Mrs. M. E. Callender, Mrs. F. A. Ehrenford, Mrs. J. G. Evans, Mrs. W. H. Headlee, Mrs. S. H. Hopper, and Mrs. W. A. Summers.

4. Editors of Society Publications. Dr. Martin Frobisher, Editor-in-Chief of the American Journal of Tropical Medicine and Hygiene, and Dr. Martin Young, Editor *pro tem*, and Dr. Louis J. Olivier, Editor of *Tropical Medicine and Hygiene News*, have maintained the high standard of these publications. THEREFORE BE IT RESOLVED that expressions of appreciation of the Society be extended to these individuals.

5. Visitors from abroad. The Society welcomes especially the visitors from abroad who attended this meeting: Dr. Jacques Cerf, Belgian Congo Health Service; Dr. Kazumine Kobari, Tokyo Municipal Komagome Hospital; Dr. Hideo Noriki, Nippon Medical School, Tokyo; Dr. Alfonso Trejos, Facultad de Medicina, University of El Salvador; Dr. Keigo Uchiyam, Nippon Medical School; Dr. Roy Jose Marques, University of Recife, Institute of Tropical Medicine; Dr. Matuosu, Japan.

There being no further business, the meeting was adjourned.

SCIENTIFIC AND SPECIAL SESSIONS

Six scientific sessions were held at which 74 papers were presented. The titles are listed below. In addition, two motion pictures were shown at two evening sessions.

At the annual dinner session, at which G. Robert Coatney presided, the Bailey K. Ashford Award was presented to Geoffrey M. Jeffery, and the American Foundation for Tropical Medicine Richard P. Strong Medal was presented *in absentia* to Herbert C. Clark. Introduced by G. Robert Coatney, President Lewis W. Hackett gave the Presidential Address, "Once Upon a Time."

SCIENTIFIC EXHIBITS

Global Attack on Disease. The extent to which preventable diseases are unconquered, and the world-wide malaria eradication program now under way. International Cooperation Administration, Washington, D.C.

Comparative Ultrastructure of the Intestinal Walls of two Nematodes: A, caninum *and* T. canis. Harry G. Browne and Amiya B. Chewdhury, Cornell University Medical College, New York, N.Y., and Calcutta School of Tropical Medicine, Calcutta, India.

MOTION PICTURES

Human Helminthiasis Throughout the World (Helmintiasis Humana en el Mundo). Francisco H. Aguilar and Carlos M. Monson M., Universidad de San Carlos de Guatemala, Guatemala City, Guatemala.

An Epidemiological Tour of Tickborne RSS Virus Areas in Eastern Europe. Telford H. Work, The Rockefeller Foundation, New York, N.Y.

1. THE TWENTY-FOURTH ANNUAL CHARLES FRANKLIN CRAIG LECTURE: *Some Impressions of World Malaria Eradication 1959.* Fred L. Soper, Director Emeritus, Pan American Sanitary Bureau.

2. *United States Participation in a Global Program for Malaria Eradication.* Roy F. Fritz, Donald R. Johnson and Eugene P. Campbell, International Cooperation Administration, Washington, D.C.

3. *Field Tests on the Control of Resistant* Anopheles *Mosquitoes in El Salvador.* Herbert F. Shoof and Willis Mathis, Communicable Disease Center, Savannah, Ga, and J. R. Austin, PASB, El Salvador.

4. *The International Municipal Water Supply Program—A Health and Economic Evaluation.* John A. Logan, Northwestern University, Evanston, Ill.

5. *Community Water Supply Development in Costa Rica.* John M. Henderson, Savannah, Ga.

6. *The Nature of the Gastrointestinal Lesions of Asiatic Cholera and its Relation to Pathogenesis: A Biopsy Study.* E. J. Gangarosa, W. R. Beisel, C. Benyajati, H. Sprinz and P. Piyarata, Walter Reed Army Institute of Research, Washington, D.C., Chulalongkorn Medical College, Bangkok, Thailand, and The Armed Forces Institute of Pathology, Washington, D.C.

7. *Eradication of Ascariasis. I. Assay in Astapa.* Francisco Biagi F. and Oscar Rodriguez, Universidad Nacional de Mexico, Mexico, D.F.

8. *Chronic Strongyloidiasis Terminating Fatally.* Herritt O. Alcorn, Jr. and Emil Kotcher, University of Louisville, Louisville, Ky.

9. *Sparganosis in Mississippi. Report of Two Cases.* T. J. Brooks, Jr., W. F. Hutchison, T. J. Safley and T. G. Ross, University of Mississippi, Jackson, Miss.

10. *Intestinal and Blood Parasitism among British West Indian Agricultural Laborers.* Henry Beye, Charles Brooks and Elizabeth Guinn, National Institutes of Health, Bethesda, Maryland.

11. Trypanosoma cruzi *in Louisiana.* A. D'Alessandro Bacigalupo and Robert C. Yaeger, Tulane University, New Orleans, La.

12. *Reductions in the Prevalence of Microfilaremia and Clinical Filariasis in the Society Islands Following Application of Control Measures.* H. N. March, J. Laigret, J. F. Kessel and Ben Bambridge, Institute de Recherches Medicales de la Polynesie Francaise, Papette, Tahiti, and University of California, Los Angeles, Calif.

13. *Observations on* Ascaris *and* Toxocara *Antigen Fractions in an Indirect Hemagglutination System.* Guillermo Pacheco and Rodney C. Jung, Tulane University, New Orleans, La.

14. *Incidence and Treatment of Nematodiases in Captive Animals.* Benedict J. Jaskoski, Loyola University, Chicago, Ill.

15. *Immunological Tolerance Studies with Mice and* Trichinella. Adam Ewert and Leroy J. Olson, University of Texas, Galveston, Tex.

16. *Serology of Visceral Larva Migrans.* Leroy J. Olson and Adam Ewert, University of Texas, Galveston, Tex.

17. *Comparative Ultrastructure of the Intestinal Walls of Two Nematodes:* Ancylostoma caninum *and* Toxocara canis. Harry G. Browne and Amiya B. Chowdhury, Cornell University, New York, N.Y. and Calcutta School of Tropical Medicine, Calcutta, India.

18. *The Anthelmintic Activity of New Formulations of Dithiazanine with Good Outpatient Tolerance.* Francisco Feebles, Jr., M. M. Brooke, Carl C. Janowsky and A. M. Perri, Communicable Disease Center, Atlanta, Ga., and Dept. of Health, Philadelphia, Pa.

19. *Clinical Evaluation of New Formulations of Tablets of Dithiazanine Iodide in Multiple Parasitosis in Guatemala.* J. G. Evans, Carlos M. Monson M. and Francisco J. Aguilar, Lilly Research Laboratories, Indianapolis, Ind. and Universidad de San Carlos de Guatemala, Guatemala City, Guatemala.

20. *The Comparative Anthelmintic Effects of Dithiazanine and Tetrachlorethylene Individually and in Combination.* Geoffrey M. Jeffery, Martin D. Young and Ben F. Wyman, National Institutes of Health, Columbia, S.C., and South Carolina State Hospital, Columbia, S.C.

21. *Efficacy of Bephenium and Tetrachlorethylene in Mass Treatment of Hookworm Infection.* Rodney C. Jung and John E. McCroan, Tulane University, New Orleans, La., and Georgia State Department of Health, Atlanta, Ga.

22. *Treatment of Enterobiasis with New Formulations of Dithiazanine Iodide.* James B. Hammond and Max McCowen, Lilly Research Laboratories, Indianapolis, Ind.

23. *A Clinical Evaluation of Four Oxyuricides.* Thomas S. Bumbalo, Lois J. Plummer and J. Roger Warner, E. J. Meyer Memorial Hospital and University of Buffalo, Buffalo, N. Y.

24. *Effects of Aqueous Iodine Solutions on Infective Stages of* Ascaris *and Hookworms on Fresh Vegetables.* Prayuth Thitasut, Tulane University, New Orleans, La.

25. Paragonimus *in South Korea: Epidemiology, Differential Diagnosis, Clinical Considerations and Treatment.* E. H. Sadun and A. A. Buck, Walter Reed Army Institute of Research, Washington, D.C., The 406th Medical General Laboratory, Tokyo, Japan, and the German Red Cross Hospital, Pusan, Korea.

26. *Strongyloidiasis in Barrio Siloé, Cali, Colombia.* Ernest Carroll Faust, Luis E. Giraldo and Gladys Caicedo, Tulane University, New

Orleans, La., and Universidad del Valle, Cali, Colombia.

27. *Some Immunologic Aspects of Trichinosis in Miniature Pigs.* Wm. Winter, J. Walter Beck, Vincent R. Saurino and Thomas A. Anfinson, University of Miami, Miami, Florida.

28. *Parasitism among Seminole Indians.* J. Walter Beck, University of Miami, Miami, Florida.

29. *Comparison of the Effectiveness of Gentian Violet, Vanquin and Dithiazanine in the Treatment of* Strongyloides stercoralis *Infections.* A. Fritsch, H. B. Shookhoff and H. M. Sterman, New York City Department of Health.

30. *Treatment of* Schistosoma mansoni *Infection in Puerto Rican Children with Lucanthone (Nilodin)—Further Experience.* H. B. Shookhoff, A. Fritsch and A. Einhorn, Health Department, City of New York and Albert Einstein College of Medicine, New York, N.Y.

31. *Ecologic Inter-relationship of* Australorbis glabratus *and* Ceratodes cornuarietis *under Field Conditions in Puerto Rico.* M. G. Radke, A. Sullivan, Jr., B. A. Dent, L. S. Ritchie and F. F. Ferguson, Walter Reed Army Institute of Research, Washington, D.C., U. S. Tropical Research Medical Laboratory and U. S. Public Health Service, San Juan, Puerto Rico.

32. *Experimentally Induced Resistance to* Schistosoma mansoni *in the Macacus Monkey. Pathologic Findings.* F. v. Lichtenberg and L. S. Ritchie, Harvard University, Boston, Mass. and Army Tropical Research Medical Laboratory, San Juan, P.R.

33. *The Action of Low Concentrations of Molluscicides on the Eggs of* Australorbis glabratus. Louis Olivier and Willard T. Haskins, National Institutes of Health, Bethesda, Md.

34. *The Miracidium of the Ochetosomatid Genera* Dasymetra *and* Ochetosoma *(Trematoda: Digenea).* Elon E. Byrd, University of Georgia, Athens, Ga.

35. *Acquired Immunity to the Rice Rat Strain of* Litomosoides carinii *in Mongolian Gerbils.* John H. Cross, Jr., J. Allen Scott and Etta Mae Macdonald, University of Texas, Galveston, Texas.

36. *Changes of Thiamine Content in Thiamine Deficient Rats with Infections of* Trypanosoma rhodiense. Ira Singer, The Rockefeller Institute, New York, N.Y.

37. Daubaylia potomaca, *a Nematode Parasite of* Helisoma trivolvis, *Transmissible to* Australorbis glabratus. Eli Chernin, Edward H. Michelson and Donald L. Augustine, Harvard University, Boston, Mass.

38. *Disinfection Tests in Grounded Aircraft Utilizing DDVP Vapor.* D. R. Maddock, H. F. Shoof and V. Sedlack, Communicable Disease Center, Savannah, Ga.

39. Falciparum *Malaria—Clinicians Beware.* Harry Most, New York University, New York, N.Y.

40. *Studies of the Exoerythrocytic Cycle in Primate Malarias. I. The Injection of Uninfected Mosquito Tissue and Other Control Procedures.* Don E. Eyles, National Institutes of Health, Memphis, Tenn.

41. *The Effect of Small Doses of Primaquine upon Malaria Infections.* Martin D. Young, National Institutes of Health, Columbia, S. C.

42. *The Potentiation of Antimalarial Activity of Chloroquine in Mice Infected with* P. berghei. L. E. Gaudeyye and G. Robert Coatney, National Institutes of Health, Bethesda, Md.

43. *Studies on the Mode of Action of Antimalarial Drugs.* Karl A. Schellenberg and G. Robert Coatney, National Institutes of Health, Bethesda, Md.

44. *Intermittent and Continuous Administration of a Combination of Amodiaquin and Primaquine (Camoprim) to Primaquine Sensitive and Non-sensitive Children.* Robert Hodgkinson and Meigs Haggerty, Parke, Davis & Co., Detroit, Mich.

45. *Trials with Amodiaquin Plus Primaquine (Camoprim) on Sporozoite Induced Chesson Strain Vivax Malaria.* K. O. Courtney, R. G. Ramsay, Robert Hodgkinson and Meigs Haggerty, Parke, Davis & Co., Detroit, Mich., and Raiford State Prison, Raiford, Fla.

46. *The Biology of a Newly Isolated Strain of* Pl. berghei *in a Rodent Host and in Experimental Mosquito Vectors.* Meir Yoeli and Harry Most, New York University-Bellevue Medical Center, New York, N.Y.

47. *The in Vitro Survival of Malaria Sporozoites.* Robert Ingram and Don E. Eyles, National Institutes of Health, Memphis, Tenn.

48. *Viruses Isolated from Cases of Epidemic Hemorrhagic Fevers of the Philippines and Thailand.* W. McD. Hammon and Gladys E. Sather, University of Pittsburgh, Pittsburgh, Pa.

49. *Growth of Nine Arthropod-borne Viruses in Hamster Kidney Tissue Culture.* Charles R. Rosenberger and William L. Pond, National Institutes of Health, Bethesda, Md.

50. *Comparative Studies on the Transmission of Japanese Encephalitis Virus by Five Species of Oriental Mosquitoes.* Douglas J. Gould, Herbert C. Barnett, William Suyemoto and Bruce F. Eldridge, Walter Reed Army Institute of Research, Washington, D.C.

51. *Dengue Types and Other Viruses Isolated from Mosquitoes of the Philippines (1956) and Thailand (1958).* W. McD. Hammon and Albert Rudnick, University of Pittsburgh, Pittsburgh, Pa.

51A. *Recent Isolations of Some New Arthropod-*

borne Viruses from India. Telford H. Work, New York, N.Y.

52. *Field Studies on Phlebotomus and Sandfly Fever in West Pakistan and Iran.* Herbert C. Barnett and William Suyemoto, Walter Reed Army Institute of Research, Washington, D. C.

53. *The in vitro Phagocytosis of* Histoplasma capsulatum. Paul R. Earl and Joseph Uscavage, University of Pennsylvania, Philadelphia, Pa.

54. *Purification of* Toxoplasma *by Sonic Vibration and Tryptic Digestion.* Yukinori Tsunematsu, University of California and Estelle Doheny Eye Foundation, Los Angeles, Cal.

55. *A Hemagglutination Test for Toxoplasmosis Using Human Erythrocytes.* William P. Lewis, John F. Kessel and Jean Onslow, Estelle Doheny Eye Foundation and University of California, Los Angeles, Cal.

56. *Immunization of Guinea Pigs Against* Toxoplasma gondii. Leon Jacobs and Majorie L. Melton, National Institutes of Health, Bethesda, Md.

57. *The Incidence of* Toxoplasma *Antibodies in Sera from Wild Mammals in Southwestern Georgia and Northwestern Florida.* Ruth H. Gogel and Alexander J. Sulzer, Communicable Disease Center, Chamblee, Ga.

58. T. gondii: *In vitro Metabolic Studies.* D. Ivler, L. T. Miller and H. A. Feldman, State University of New York, Syracuse, N.Y.

59. *Results of Fluorescence Inhibition and Dye Tests for Toxoplasmosis in Human and Animal Sera.* R. K. Carver and Morris Goldman, Communicable Disease Center, Chamblee, Ga.

60. *Infective Trypanosomes in Tsetse Fly Tissue Culture.* William Trager, Rockefeller Institute for Medical Research, New York, N.Y. and West African Institute for Trypanosomiasis Research, Nigeria.

61. *The Effects of Certain Polyoxyethylene Ethers on Experimental Infections with African Pathogenic Trypanosomes.* Frans C. Goble and James L. Boyd, CIBA Pharmaceutical Products, Summit, N. J.

62. *Metabolic Effects produced in* Trypanosoma lewisi *by the Antibody Ablastin.* Tulio Pizzi and William H. Taliaferro, University of Chicago, Chicago, Ill.

63. *Enzyme Profile of* Vibrio comma *and El Tor Vibrio.* Harry Seneca, O. K. Troc and Peggy Milner, Columbia University, New York, N. Y.

64. *Suppressive Therapy of Amebiasis in Primates.* Hamilton H. Anderson and Arseny K. Hrenoff, University of California, San Francisco, Cal.

65. *Observations on Etiology of Diarrheal Disease in Arizona.* M. H. Goodwin, Jr., Don C. Mackel, Robert S. Ganelin, Robert E. Weaver and Fred J. Payne, Communicable Disease Center, Phoenix, Arizona.

66. *Studies on the Gram-negative Anaerobic Bacillus Used in the Shaffer-Frye Medium.* Joseph J. McDade and James G. Shaffer, The Chicago Medical School, Chicago, Ill.

67. *Microfluorimetric Evidence of an Antigenic Difference between* Entamoeba histolytica *and* Entamoeba hartmanni. Morris Goldman, Communicable Disease Center, Chamblee, Ga.

68. *Comparative Findings of Small and Large Race* Entamoeba histolytica *in Proctoscopic Aspirates and in Stools.* William H. Shales, James G. Shaffer and Alex Stahl, The Chicago Medical School, Chicago, Ill.

69. *Studies on the Ingestion of Altered Bacterial Structures and of Bacteria by* Entamoeba histolytica *in the Shaffer-Frye Medium and in Modified Shaffer-Frye Medium.* James G. Shaffer and Iris D. Key, The Chicago Medical School, Chicago, Ill.

70. *Some Quantitative Data on Growth of Amebas from Single-cell Isolations in Microcultures with a Method of Transferring the Progeny to Medium in Test Tubes.* Charles W. Rees, Iris D. Key and James G. Shaffer, The Chicago Medical School, Chicago, Ill.

71. *The Effect of Surface-active Agents upon* Entamoeba histolytica *in Culture.* Richard E. Reeves, Louisiana State University, New Orleans, La.

72. *Studies on Monoprotozoal Cultures of* Entamoeba histolytica. Chia-tung Pan, Harvard University, Boston, Mass.

73. *Pathogenesis of Fatal Diarrheal Disease in Malnourished Children.* Donald Feldman, Carlos Tejada, Warren Hoffert, Virginia Pierce, E. B. Yawn, Nevin Scrimshaw and Gustave J. Dammin, Harvard University, Boston, Mass. Institute of Nutrition of Central America and Panama (INCAP), Guatemala, Hospital Roosevelt of Guatemala, and the Miami Regional Laboratory of the Florida State Board of Health.

74. *Protection of Human Enteric Pathogens against Chlorination in Free-living Nematodes Isolated from Water Supplies.* Shih L Chang, Gerald Berg, Norman A. Clarke and Paul A. Kabler, Robert A. Taft Sanitary Engineering Center, Cincinnati, Ohio.

BOOKS RECEIVED

Annual Review of Entomology, by Edward A. Steinhaus and Ray Smith, Ed'rs. 4th edition, 467 pages. Palo Alto, Calif. Annual Reviews, Inc., 1959. $7.00.

Immunization Information for International Travel, Public Health Service Public'n. No. 384. 77 pages, ill. Washington, D.C., Gov't. Printing Office, 1959. $0.30.

Physicians for a Growing America. (Report of the Surgeon General's Consultant Group on Medical Education), Public Health Service Publication. No. 709. 1st edition, 95 + xiv pages, maps. Washington, D.C., Gov't. Printing Office, 1959. $0.60.

Microbial Variation, by V. D. Timakov, Ed'r. (Translated from the Russian by G. H. Beale). 1st edition, 202 + x pages, ill. New York, N.Y., Pergamon Press, 1959. $6.50.

Blindness in West Africa, by F. C. Rodger, M.D., Ch.M., D.O.M.S., F.R.S.T.M.&H. 1st edition, 262 pages + xiv, ill. London, H. K. Lewis & Co., Ltd. (for the Royal Commonwealth Society for the Blind), 1959. £3.10.0d.

BOOK REVIEWS

The Ecology of the Medical Student. Report of the Fifth Teaching Institute, Association of American Medical Colleges, by HELEN H. LEE AND ROBERT J. GLASER, editors. 262 pages, illustrated. Evanston, Ill., Association of American Medical Colleges, 1958. Cloth $5.00, paper $2.00.

This book reports the proceedings of the Fifth A.A.M.C. Teaching Institute held in Atlantic City, N. J. in October, 1957. These Institutes have been supported by grants from the Commonwealth Fund and the Public Health Service and the first three were concerned with basic medical sciences. Their value cannot be in doubt, for several medical schools have revised their curricula in the light of the topics discussed. But there are many factors other than those listed in the curriculum that bear upon the education of a doctor. The main topic of the Fourth Institute was the selection of medical students and that of the Fifth is the environment of the school with particular reference to the environmental factors which affect the development of professional attitudes and human and social values. The participants were representatives from 99 medical schools; 86 in the United States, 11 in Canada, 1 in Puerto Rico, and 1 in Britain. Among them were a number of psychologists and social scientists whose contributions to the subject are stimulating.

Almost everyone who has experienced the rigors (and the joys) of becoming a doctor will express opinions upon medical education. Valuable though these may be there is a lack of comparative data upon which to base sound views, and past efforts to accumulate such data have been few. In the present report data were collected by means of three questionnaires sent to each of the participating medical schools during the months prior to the Institute. The responses were collected, analyzed, and presented to small groups at the Institute, primarily for discussion and to permit the interchange of ideas rather than to serve as a basis from which conclusions might be drawn. Much of the material reported is new and the contributors are agreeably objective.

Medical schools differ greatly from one another but each is an exceedingly complex social environment and its influence upon the subsequent attitudes and behavior of its alumni is subtle. One gains an impression that some of the forces at work are measurable. It is to be hoped that this represents only a beginning; that in the future further efforts involving other methods of data collection will lead to a clearer understanding of the factors affecting medical education and ultimately the practice of medicine. Teachers of medicine will find this volume provocative and illuminating, and will wish for more.

JOHN C. S. PATERSON

1959—DDT. The Insecticide DDT and its Significance. Edited by PAUL MULLER, Basel. **Volume II. Human and Veterinary Medicine.** Edited by S. W. SIMMONS, Atlanta, Ga., U.S.A. Authored by W. J. HAYES, Savannah, Georgia, U.S.A.; S. W. SIMMONS, Atlanta, Ga., U.S.A.; and E. F. KNIPLING, Washington, D. C., U.S.A. 570 pp, 63 figures and 65 tables. Basel and Stuttgart, Birkhäuser Verlag. 1959. Fr. 66—(DM 66—).

The second tome of this serial treatise on DDT adds three more chapters to the five in the first volume. These are entitled 1) Pharmacology and Toxicology of DDT, 2) The Use of DDT Insecticides in Human Medicine, and 3) The Use of DDT in Veterinary Medicine. They are contributed, respectively, by the eminent authorities in those subjects listed above.

The first of these, Chapter VI in the series, is by Dr. W. J. Hayes, Jr., who, with his associates at the Communicable Disease Center Technology Laboratory in Savannah, Georgia, has been studying and evaluating the biological effects of DDT and other modern insecticides for many years. Dr. Hayes' contribution is a meticulously written summary of, and commentary upon, the literature (685 references cited) pertaining to his chapter title, with tables, graphs, and illustrations to clarify or condense its content. His chapter constitutes nearly one-half of the book, 250 of 570 pages, and deals with such items as clinical indications of DDT intoxication in man and lower animals, dose-mortality relationships referable to single and multiple doses and to oral, respiratory, and dermal applications. He discusses the pathologic changes in various tissues in association with the magnitude and number of doses, and with different types of exposure.

Information concerning the physiologic and biochemical aspects of DDT metabolism is treated on the same broad scale, quantitatively where possible. Thus, the distribution and storage of DDT and its metabolic products in various tissues; the effects of these compounds on digestive, respiratory, excretory, circulatory, and nerve functions; and the phenomena of detoxification and the development of tolerance in certain animal phyla are discussed. The same chapter contains a section on chemicals and drugs known to have antidotal or other antagonistic effects on DDT.

A separate subchapter is devoted to observations on the toxicity of DDT for man. These are mainly clinical and are presented according to the mode of their derivation. The first of these concerns information developed from various types of experimental exposure. In this connection, it is most regrettable that the lead time from the completion of the book manuscript (no references given since 1954) to its publication in 1959 has been so great that it has prevented including results of the comprehensive experimentation in this field by Dr. Hayes and his collaborators.

Further information concerning human intoxication by DDT is available from reports of accidental poisoning; these are reviewed in detail. The author points out that these unfortunate mishaps are frequently complicated by simultaneous exposure to other suspect substances, including solvents, emulsifiers, diluents, etc., and that much caution should be exercised in arriving at conclusions regarding primary toxic roles under such circumstances. In this connection, he records his distrust of certain contentions made, about a decade ago, with reference to the etiologic role of DDT in causing or predisposing toward certain diseases of unknown origin.

The last source of information regarding the effect of DDT on human health comes from the observation and examination of individuals who have sustained prolonged and intimate incidental exposures to the compound. These include members of DDT dusting or spraying crews, persons engaged in the manufacture of DDT, and individuals on whom DDT dust has been applied as a mass delousing procedure to prevent epidemic typhus fever. Published findings in this field are scrupulously reviewed by the author who concludes that no major functional or organic disturbance was shown to be due solely to these types of exposure to DDT.

The occurrence of DDT in human foods of animal origin (dairy products, meat, and eggs), of vegetable origin (fruits, leafy vegetables, and grains), and in water has long been of concern to private investigators and various governmental agencies having regulatory responsibilities or scientific interests concerning health, agriculture, and food quality. This has occasioned much research on the analytic determination of DDT content in or on food, its significance, and on means of removing or detoxifying DDT residues before human consumption. These are reviewed by the author in detail, and the conclusion is reached that these do not now constitute public health hazards under prevailing conditions of ordinary use. Information regarding current regulatory policies and procedures is presented.

The last portion of Dr. Hayes' contribution is concerned with the hazards of DDT and its accompanying chemicals to domestic animals and to wildlife. For the former, this appears to be minimal; for the latter, its effect upon arthropods, other invertebrates, and under certain circumstances on fish may be marked. Birds and mammals appear to be less affected, even though they may feed extensively on DDT-contaminated wildlife and vegetation.

In summary, Dr. Hayes has authored an exceedingly interesting and comprehensive survey of literature concerning the pharmacologic and toxicologic aspects of DDT and related chemicals. His interpretation of what these areas encompass is very broad; indeed, he has included much material which is only remotely, if at all, relevant to the title of his section. However, this makes it an even more useful compilation, interestingly presented and thoughtfully analyzed. It will be of great assistance to many concerned in the use of DDT, and in understanding its mode of action and its potential hazards.

The second chapter (Chapter VII) is a comprehensive account (223 pages plus 668 references) of experience in the use of DDT in reducing human disease or discomfort resulting in part or entirely

from the bites of arthropods. After a brief introductory statement summarizing the collective significance of DDT in terms of health, and of economic and social benefits, the succeeding sections are concerned individually with specific or group arthropod transmitters of disease (mosquitoes, flies, lice, fleas, hemipterous bugs, ticks, mites) or sources of nuisance and discomfort (bedbugs, roaches, fleas, flies, and mosquitoes). These vectoral entities are considered first in reference to particular diseases (*e.g., mosquitoes:* malaria, filariasis, dengue, yellow fever, viral encephalitis) or to pestiferousness and, secondly, on a geographic basis (*e.g., malaria:* Italy, Sardinia, Corsica, Spain, Portugal, Netherlands, Yugoslavia, Greece, Turkey, Cyprus, the Levantine countries, Iran, Afghanistan, Pakistan, India, Ceylon, Burma, Thailand, Malayan Federation, Associated States of Indochina, Republic of China, Philippine Islands, Indonesia, other areas in the South Pacific, Egypt, Morocco, Liberia, Nigeria, Kenya, Uganda, Tanganyika, Belgian Congo-Katanga, Ruanda-Urundi, Southern Rhodesia, Union of South Africa-Transvaal, Swaziland, Madagascar, Mauritius, U.S.A., Mexico, Puerto Rico, Guatemala, Nicaragua, Costa Rica, Panama, Colombia, Venezuela, Trinidad, British Guiana, French Guiana, Brazil, Ecuador, Peru, Bolivia, Chile, and Argentina). Demographic, vector, cost, and evaluation data are shown. Thus, knowledge of the use and results of DDT application against many types of arthropods all over the world, up to and including 1954, is reviewed in remarkable detail.

Two other sections by the same author discuss: 1) the use of DDT in the disinsectization of aircraft and ships; and 2) types and mechanisms of resistance to DDT, and the significance and future outlook regarding this phenomenon.

The final chapter of Volume II (Chapter VIII of the series) is 57 pages in length, has 203 references, and is abundantly illustrated. It deals with the use of DDT in veterinary medicine, and consists of five brief sections and a single longer one.

The former are concerned with: 1) the comparative toxicity of DDT to livestock and domestic animals (cats are most often affected due to their practice of frequent fur-licking); 2) the greater health hazard to livestock from materials used as diluents of DDT, especially in dips; 3) the formulation, and 4) the application, of DDT solutions, emulsions, suspensions, and dusts used in veterinary medical practice; 5) environmental (residual) applications versus direct host treatment.

The longest and final section deals with the use of DDT against specific arthropod vectors of domestic-animal and livestock disease. It emphasizes the fact that insecticidal methods should be accompanied where feasible by practicable sanitary measures to prevent vector breeding or to exclude arthropods from domestic animal shelters. The specific and group types of livestock disease vectors mentioned, together with procedures advocated for their control with DDT, include the following: house flies; stable flies; tse-tse flies; horse flies and deer flies; black flies or buffalo gnats; the horn fly and the buffalo fly; mosquitoes of various kinds; sheep "ticks" and other Hippoboscidae; various species of true ticks; cattle, hog, horse, and poultry lice; fleas; bed-bugs; mites; and various species of fly larvae which parasitize livestock.

The broad and multiple spectra of interest in "DDT, Volume II, Human and Veterinary Medicine" recommend it to a vast diversity of readers: administrative, scientific, industrial, professorial, and sanitarian. This will include physicians, veterinarians, biologists, chemists, drug manufacturers, insect-control specialists and their respective subspecies such as preventive medicine and public health practitioners, pharmacologists, toxicologists, pathologists, biochemists, livestock sanitarians, medical entomologists, and malaria control workers.

JUSTIN M. ANDREWS

The Human Integument, by STEPHEN ROTHMAN, editor, with 13 contributors. 1st edition, 260 pages, illustrated. Washington, D. C., American Association for the Advancement of Science, 1959. $6.75.

A lucid, informative and valuable monograph containing papers presented by 13 authors at the December, 1957, meeting of the American Association for the Advancement of Science. From his unique background as author of the classic work on biochemistry and physiology of the skin, Rothman, the editor of the present volume, perceptively selected a group of outstanding partici pants. As the editor notes, it is not sufficient that research be successful; the news of this success must be disseminated. Most, but not all, of the authors are dermatologists actively engaged in basic laboratory research. With one foot in the laboratory and the other in the clinic the authors present their studies so as to provide new and refreshing insights into the altered physiology of diseases of the skin. There is much that is basic and much that is clinical. The success of the correlation between the two could well serve as a model for future efforts.

The volume is divided into the following four sections: The integument as an organ of protection; Circulation and vascular reaction; Sebaceous gland secretion; and, Pathogenetic factors in premalignant conditions and malignancies of the

skin. It is impossible to review each chapter except to extol their uniform excellence and their outstanding value to all who would deal with the human integument.

HARVEY BLANK

Annual Review of Entomology by EDWARD A. STEINHAUS, Editor, and RAY F. SMITH, Associate Editor, University of California. Volume 4, 467 pp. Palo Alto, California, Annual Reviews, Inc. 1959. $7.00.

This fourth volume of reviews of selected entomological subjects maintains the high standards and objectives of earlier volumes. Scientists from seven countries contribute 19 papers and each is a pertinent current review of worldwide studies on the subject. The reviewer would draw the particular attention of readers of this journal to three chapters: "Culture of Insect Tissues" by Day and Grace, "Fleas and Diseases" by Jellison, and "Insects and the Epidemiology of Malaria" by Russell.

The paper on insect tissue culture is most timely as it summarizes historical and current studies which have not been brought together previously. Current interest in the subject is dictated by the need for methods of maintaining pathogenic agents in vector tissues without intermediate passage through vertebrate or plant hosts. Factors which have limited the success of attempts to culture insect tissues are summarized as are the avenues which may lead to future success. The technical aspects of the problem and variety of insect tissues which have been studied are well covered.

The paper on "Fleas and Disease" is more inclusive than the title indicates. The historical development of systematic and biological studies of fleas is summarized. In the treatment of specific diseases such as plague, tularemia, murine typhus and filariasis consideration is given to the relative importance of flea transmission as compared with other vectors. While one might disagree with interpretations which lead to the conclusion that mites are of importance as vectors of murine typhus, the comparisons and contrasts are most worthwhile. The inclusion of considerations of the direct effect of fleas on their hosts and of miscellaneous diseases and microorganisms associated with fleas makes this a most complete review.

The paper on "Insects and the Epidemiology of Malaria" is briefer than might be expected from the title and subject. However, the author succinctly summarizes the principal contributions in this field, and current trends. Two master tables give key references to literature on the *Anopheles* malaria vector species by countries of the world and the current status of malaria in these countries.

The other 16 papers in this volume are devoted to: insect blood cells, pheromones (ectohormones) in insects, insect pigments, taxonomic problems with closely related species, ecology of Cerambycidae, biology of aphids, the biology of parasitic Hymenoptera, bioclimatic studies with insects, ethological studies of insect behavior, experimental host-parasite populations, biological control of weeds with insects, microbial control of insect pests, the mode of action of insecticides, biological assay of insecticide residues, deciduous fruit insects and their control, and seed treatment as a method of insect control. Each is an excellent summary of the respective subject.

W. C. REEVES

CORRECTION

Substitute the following for Table 3 on page 626 in the article "Investigation of parasitic infections in the Central area of Philadelphia" by Daniel Weiner, M. M. Brooke and A. Witkow in Vol. 8, No. 6, pages 625–629.

TABLE 3

The amebic and helminth prevalence rates in groups of school children

Prevalence index	Puerto Rican (167 children) %	Non-Puerto Rican	
		White (169 children) %	Negro (49 children) %
Amebic (APR)..	40.7	20.7	32.7
Helminth (HPR).....	70.7	3.0	4.1

NOTICE TO CONTRIBUTORS

The Journal welcomes papers which contribute to the purpose of the American Society of Tropical Medicine and Hygiene. This is the advancement of knowledge of tropical and subtropical medicine and hygiene through investigation, prevention, treatment and control of diseases and deficiencies. It draws upon medicine, biology, public health, nursing, engineering and allied fields of research and application.

The American Journal of Tropical Medicine and Hygiene combines the American Journal of Tropical Medicine and the Journal of the National Malaria Society and therefore has a special concern with the investigation, therapy and control of malaria in all parts of the world, as well as a broad interest in general parasitology and arthropod-borne diseases.

Papers are ordinarily published in the order of reception, which will entail a certain delay in their appearance because of the contractual limitation on the number of journal pages available in any year. Accepted articles which raise no question of scientific priority but for which immediate publication seems important will be inserted in the next available issue of the journal if the author or sponsoring institution will pay the cost, estimate of which may be obtained from the editor. The insertion of such articles does not affect publication of other manuscripts awaiting their turn, since the inserted articles constitute additional pages in the volume without additional cost to the subscriber.

Publication will be facilitated if the following suggestions are followed:

1. *Manuscripts* should be typewritten in *English* using *double spacing* on *one side* only of 8½ x 11 inch paper and submitted *in duplicate*. Double-spacing should be used throughout the manuscript including the list of references, footnotes, and quotations.

2. The author should supply, on a separate sheet, a short title not over 50 letters for use as a running head. Also on separate sheets type (a) the authors' names and affiliations; (b) tabular matter; (c) case histories; (d) quotations; (e) formulas and other subsidiary matter in the text; (f) footnotes; (g) references; (h) legends for illustrations; (i) displayed formulas occupying more than 1 line of type, even if using the same size type as the main text.

3. Legends should be separate and not attached to, or written on, the illustration copy.

4. Positions for tables and figures in the text may be indicated on the margin of the text pages.

5. All papers should end with a brief summary.

6. *Tables* should be typed on separate sheets and be serially numbered in Arabic numerals.

7. *Figures* and *photographs* should be protected with cardboard to avoid creasing in the mails. Only glossy prints can be used. Line drawings and graphs should be made with India ink and submitted on white (not cross-section) paper. Colored illustrations can be reproduced only at the expense of the author.

8. References should be cited by consecutive numbers in the text, and so numbered and listed in the list of references at the end of the article, without regard to alphabetical arrangement. References should be written as follows:

> 1. Reed, L. J., and Muench, H., 1938. A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.*, **27**: 493–497.

9. *Proprietary names* of drugs or chemicals registered by commercial pharmaceutical firms may not be used in the text if there is a generic name available in the U. S. Pharmacopoeia, New and Nonofficial Remedies of the A. M. A., or the latest editions of standard medical dictionaries. In certain cases where the generic name is relatively unknown, the registered name may follow it in parentheses in the title, at the first citation in the text, and in the summary.

10. **N. B. The address to which proofs are to be sent** should be indicated at the top of the first page of the manuscript. Delay will result if proofs must be forwarded.

Estimating costs of reprints. Where the cost of reprints must be known in advance of publication in order to secure prior approval for their purchase, the Editor will be glad to furnish an estimate of the number of pages which the article will occupy in the Journal.

DIFCO

Diagnostic Reagents
for the Determination of

ANTISTREPTOLYSIN TITERS

Bacto-Streptolysin O Reagent is a standardized reagent recommended as an aid in the diagnosis of Rheumatic Fever, Glomerulonephritis and other diseases associated with Group A streptococcal infections.

SENSITIVITY TESTS

The significance of determining bacterial sensitivity is accentuated by the discovery of increasing numbers of antibiotics, sulfonamides, and other chemotherapeutic agents and the development of resistant organisms to one or more of these compounds. Sensitivity or resistance is easily determined with Bacto-Sensitivity Disks.

HETEROPHILE ANTIBODIES

The differential diagnosis of Infectious Mononucleosis requires the use of standardized heterophile reagents:

Bacto-Guinea Pig Kidney Antigen, Desiccated and Suspension
Bacto-Beef Cell Antigen, Desiccated and Suspension

BLOOD COAGULATION

Prothrombin determinations require accurately prepared interstandardized reagents:

Bacto-Thromboplastin
Bacto-Prothrombin 2 Stage Reagent
Bacto-Prothrombin Free Beef Plasma
Bacto-Prothrombin Free Rabbit Plasma
Bacto-AC Globulin
Bacto-Sodium Chloride 0.85 Per Cent
Bacto-Sodium Oxalate Solution 0.1 Molar
Bacto-Calcium Chloride Solution 0.02 Molar

LIVER DISEASE

Differential diagnosis of hepatic disturbances is aided by standardized reagents:

Bacto-Cephalin Cholesterol Antigen
Bacto-Thymol Turbidity Reagent
Bacto-Kingsbury Turbidity Standards
Bacto-Thromboplastin and other Prothrombin Reagents

RENAL FUNCTION

Phenolsulfonphthalein Ampules, Difco is a carefully prepared injectable recommended for use in determining renal excretion rate.

HEMAGGLUTINATION OF RED BLOOD CELLS

Accurately determined by the use of:

Bacto-Trypsin 1% for Hemagglutination
Bacto-Hemagglutination Buffer
Trypsin, Difco, 1:250
Bacto-Bovine Albumin 30%
Bacto-Phytohemagglutinin

DIFCO LABORATORIES

DETROIT 1, MICHIGAN